FUNCTIONAL STUDIES ON THE PEPTIDYL TRANSFERASE CENTER OF MAMMALIAN MITOCHONDRIAL RIBOSOMES

By

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Abbreviations

A_{260}	absorbance at a wavelength of 260 nanometers
ATP	adenosine-5'-triphosphate
CAP	chloramphenicol
cpm	counts per minute
dalton	the mass of 1 hydrogen atom
DOC	sodium deoxycholate
dpm	disintegrations per minute
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetate
g	gravity
GTP	guanosine-5'-triphosphate
h	hour
M	molar (mole/litre)
min	minute
mM	millimolar (millimole/litre)
mRNA	messenger RNA
poly U	poly uridylic acid
RNA	ribonucleic acid
rRNA	ribosomal RNA
S	sedimentation coefficient (Svedberg units)
sec	second
Tris	tris(hydroxymethyl)aminomethane

tRNA	transfer RNA
μ	ionic strength

Definitions

"native" subribosomal particles - subribosomal particles obtained directly from lysed mitochondria, after sucrose density gradient centrifugation, as by-products of mitochondrial monosome preparations.

"derived" subribosomal particles - subribosomal particles obtained by dissociating mitochondrial 55S monosomes in a high salt buffer.

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Ribosomes isolated from mammalian mitochondria have unique physical-chemical properties when compared to prokaryotic and eukaryotic ribosomes. Although their molecular weight (2.8 million daltons) is close to that of Escherichia coli ribosomes, their sedimentation coefficient is 55S compared to 70S for those from E. coli and 80S for those from the cytoplasm of eukaryotic cells. This sedimentation behavior reflects their unusual composition. They have a greater proportion of protein, 70%, than the 35 to 55% normally encountered in ribosomes. The ribosomal RNAs, which are smaller than those found in other ribosomes, constitute only 30% of the total mass. It seems likely that a ribosome having these unique physical properties would also exhibit unique functional properties.

In this work, some functional characteristics of mitochondrial ribosomes were investigated. The overall mechanism of protein synthesis appeared to be similar to that of other ribosomes as assessed in cell free systems involving poly-uridylic acid-directed phenylalanine polymerization, guanosine triphosphate (GTP)-binding, and peptidyl transferase activity. For protein synthesis, mitochondrial ribosomes required, in addition to amino acids, energy (adenosine triphosphate and guanosine triphosphate), template, transfer ribonucleic acid, and soluble factors. In the poly-uridylic acid-directed assay for protein synthesis, mitochondrial ribosomes were able to utilize unpurified E. coli elongation factors. In the GTP-binding assay, [^{3}H]GTP was observed to bind to mitochondrial ribosomes with the same degree of efficiency as it binds to E. coli ribosomes, which suggests the presence of a bound elongation factor on the isolated mitochondrial ribosomes analogous to the bacterial elongation factor G.

The peptidyl transferase activity of mitochondrial ribosomes was investigated in depth using the modified fragment reaction. The peptidyl transferase activity was localized on the large subunit. The activity is diminished by 75% when the ribosomes are salt-washed in buffers which do not affect the activity of E. coli ribosomes. The mitochondrial ribosomal activity is therefore more labile than that of E. coli ribosomes.

Several antibiotic inhibitors were used as specific probes of the peptidyl transferase activity of mitochondrial ribosomes. The antibiotic susceptibility of 55S ribosomes was compared to that of E. coli ribosomes and bovine cytoplasmic ribosomes. Mitochondrial ribosomes appeared to be sensitive to several inhibitors of prokaryotic specificity, such as chloramphenicol and the streptogramins. But they showed significantly lower susceptibilities to the macrolides and the lincosamines, also inhibitors of prokaryotic protein synthesis. Ten- to 700-fold higher concentrations of these latter drugs were required to inhibit the peptidyl transferase activity of mitochondrial ribosomes relative to that required for typical bacterial ribosomes. From earlier studies, it was known that mitochondrial ribosomes were not sensitive to inhibitors specific for protein synthesis occurring on ribosomes obtained from the cytoplasm of eukaryotic organisms. The peptidyl transferase center of mitochondrial ribosomes is therefore discriminated from those of both prokaryotic and eukaryotic cytoplasmic ribosomes on the basis of its response to antibiotic probes.

CHAPTER 1
INTRODUCTION

Physical-Chemical Properties of Mammalian Mitochondrial Ribosomes

Ribosomes of unique physical-chemical properties were discovered in mammalian mitochondria by O'Brien and Kalf eight years ago (O'Brien and Kalf, 1967a; O'Brien and Kalf, 1967b). Intact rat liver mitochondria were able to incorporate added [^{3}H]leucine into acid-insoluble protein in the absence of cell sap or added energy in the form of ATP. Under these conditions, the microsomal system does not synthesize proteins. The treated mitochondria were lysed and the material sedimenting at 230,000 x g was collected. Analysis on sucrose density gradients revealed that the radioactivity was localized on particles sedimenting at 55S. Subsequently, the ribosomal nature of these 55S particles was confirmed by experiments in which the monosome dissociated reversibly into 28S and 39S subunits under conditions of low Mg^{2+} and high ionic strength (O'Brien, 1971). Swanson and Dawid (1970) provided additional confirmation by showing protein synthesizing activity in a poly U dependent system by the combined subunits of 55S particles of mitochondria from *Xenopus laevis* eggs.

The existence of mitochondrial ribosomes with a sedimentation coefficient close to 55S has been observed for other mammalian organisms (reviewed in: Borst and Grivell, 1971; O'Brien and Matthews, 1975). Having also been found in Xenopus laevis (Swanson and Dawid, 1970), shark (O'Brien, 1972), and locust (Kleinow et al., 1971), they probably appear in all multicellular animals.

The low sedimentation coefficient of these ribosomes is not due to a low molecular weight as was once generally assumed (Borst and Grivell, 1971). Indeed, they have molecular weights of 2.8×10^6 daltons, close to the value of 2.7×10^6 daltons for E. coli ribosomes (Hamilton and O'Brien, 1974; de Vries and Kroon, 1974). This is due instead to a very low buoyant density, corresponding to an abnormally high protein content (Hamilton and O'Brien, 1974; Sacchi et al., 1973; O'Brien et al., 1974; de Vries and Kroon, 1974). O'Brien has found a buoyant density of 1.40 g/cc for the 55S form as opposed to 1.58 for extramitochondrial ribosomes and 1.63 to 1.65 for most bacterial ribosomes. This corresponds to a protein content of 70% which is considerably more than the 35 to 55% found in other ribosomes.

Examination of the ribosomal protein content by 2 dimensional polyacrylamide gel electrophoresis reveals that mitochondrial ribosomes contain many more ribosomal proteins, 90 (David Matthews, personal communication), than the ribosomes of E. coli or eukaryotic cytoplasm

which contain 55 (Kaltschmidt and Wittmann, 1970; Kurland, 1972) and 70 (Wool and Stöffler, 1974) respectively. The role that the extra ribosomal proteins play in the function of mammalian mitochondrial ribosomes is not known.

The physical-chemical properties of animal mitochondrial ribosomes differ remarkably from those of protists and fungi (reviewed in O'Brien and Matthews, 1975). Functional phylogenetic differences between mitochondrial ribosomes of fungal and mammalian origins were proposed by Linnane and his group (Clark-Walker and Linnane, 1966; Lamb et al., 1968; Towers et al., 1972; Towers et al., 1973). Their conclusion was supported by the observation of differential inhibition caused by certain antibiotic inhibitors of protein synthesis when intact mitochondria isolated from yeast and rats were used. These results were challenged by Kroon and de Vries (1971) who showed that in many cases the insensitivity was due to impermeability of the mitochondrial membrane to the antibiotics. Conclusive evidence for possible phylogenetic differences among mitochondrial ribosomes rested on the analysis of the mechanism of protein synthesis of the isolated particles.

Biogenetic Properties of Mitochondria

The existence of mitochondria in the cytoplasm of eukaryotic cells has aroused new interest in the past few years, especially after the discovery that these organelles

contain their own DNA, RNA and protein synthesizing apparatus (reviewed in Ashwell and Work, 1970). Several theories have been advanced to explain their presence in the early eukaryotic type cell, the most popular of which is the theory of endosymbiosis. This theory suggests that mitochondria were once symbiotic bacteria which invaded eukaryotic cells and, through evolution, became dependent on the host cell genome for some of their vital functions while, conversely, the host cell became dependent on products originating from the mitochondria (reviewed in Ashwell and Work, 1970). This theory is supported by investigators, who find many similarities between the genetic apparatus of mitochondria and that of typical prokaryotes.

Alternatively, mitochondria may have originated through "cluster-cloning" (reviewed in Bogorad, 1975). According to this hypothesis, the cell's genetic material was partitioned off into gene clusters. Each cluster and the protoplasm immediately around it were enclosed by membranes to form mitochondria, chloroplasts and nuclei. These compartments would have the capacity to reproduce themselves. Because of gene segregation in the original genome, gene products from different clusters may be needed to complete functional units such as the electron transport system in mitochondria. According to this hypothesis, genes would have been separated into the compartments early in the formation of eukaryotic cells,

rather than having been transferred from one compartment to another at some later time.

The plasmid theory proposed by Raff and Mahler (1972) is a variant of the cluster-clone hypothesis. According to this theory groups of genes in the form of plasmids would become detached from the nucleus. Then membranes would develop around them to form organelles, which would have the capacity to reproduce themselves.

Protein Biosynthesis in Mitochondria

Of the many proteins found within mitochondria, there are probably not more than 12 encoded by the mitochondrial DNA (reviewed in Schatz and Mason, 1974). In fungi 8 products have been identified. They are 3 subunits of the cytochrome oxidase (Mason and Schatz, 1973), 4 subunits of the oligomycin-sensitive ATPase (Tzagoloff and Meagher, 1972; Ebner *et al.*, 1973) and 1 subunit of cytochrome b (Weiss *et al.*, 1971; Weiss, 1972). The mitochondrial ribosomal proteins are among those synthesized in the cytoplasm of eukaryotic cells (Küntzel, 1969b). These ribosomal proteins are mitochondrial specific and differ from those found in cytoplasmic ribosomes (Küntzel, 1969a, and David Matthews, personal communication).

The biogenetic system in mitochondria thus exists primarily for the synthesis of a few proteins which must be extremely important for the survival of cells. Evidence for a tight coordination between mitochondrial and

cytoplasmic protein synthesis is slowly appearing. Exactly how they depend on each other is not yet known. The mitochondrial products seem to be needed for the correct integration of the cytoplasmically made proteins into complexes in the inner membrane (Schatz and Mason, 1974).

Mitochondrial DNA

All eukaryotes contain specific mitochondrial DNAs which are not encoded by nuclear DNA. They occur in small, circular duplex forms, their size being considerably larger in mitochondria from fungi, 15 to 30 μ, than in mammalian mitochondria, 5 μ (Borst and Flavell, 1972). In mammals, mitochondrial DNA has a limited coding capacity. Hybridization studies show it codes for mitochondrial rRNA and tRNA and may code for a few mitochondrial proteins as described above.

At most, 20 mitochondrial tRNAs have been found to hybridize with the mitochondrial DNA in yeast. In animals fewer complements have been found; for example, 15 in Xenopus (Dawid, 1972) and 12 in HeLa cells (Aloni and Attardi, 1971a; Wu et al., 1972). These numbers are considerably less than the minimum necessary to read the possible 61 codons even allowing for maximal wobble. Various possibilities arise: a) not all of the mitochondrial tRNAs have been found, b) some of the tRNAs found in the mitochondria are coded by nuclear genes and

therefore do not hybridize with the mitochondrial DNA, or c) not all the codons are used in the mitochondria. This last possibility is favored by Costantino and Attardi (1973).

Apparently both strands of DNA are transcribed for information. Nass and Buck (1970) have demonstrated that in mitochondria from rat livers, two tRNAs hybridized to the heavy strand of DNA, and two to the light strand. Even more convincing are Attardi's genetic maps showing hybridization of ferritin-labelled tRNAs from HeLa cells to both strands of the mitochondrial DNA (Wu et al., 1972).

Mitochondrial RNA

Aloni and Attardi (1971b) have demonstrated that there is symmetric transcription of mitochondrial DNA. The RNA products transcribed from the heavy strand of mitochondrial DNA had a relatively long half life when compared to those made from the light strand. Symmetric transcription of DNA was confirmed by several laboratories using RNA polymerase from E. coli to synthesize mitochondrial RNA in vitro (Schafer et al., 1971; Tabak and Borst, 1970). In these studies, the product made from the light strand was predominant.

Several investigators have succeeded in isolating mitochondrial RNA polymerases from a number of organisms (Gadaleta et al., 1970; Küntzel and Schafer, 1971;

Scragg, 1971; Tsai et al., 1971; Wintersberger, 1970; Wu and Dawid, 1972). Recently Scragg (1974) used isolated yeast RNA polymerase to transcribe yeast mitochondrial DNA. He translated the RNA product in a cell free system using E. coli ribosomes and soluble factors. He obtained several labeled polypeptides but these have not yet been identified as specific mitochondrial products.

For the most part, RNA polymerases isolated from mitochondria appear to be sensitive to rifampicin (Gadaleta et al., 1970; Küntzel and Schafer, 1971; Scragg, 1971). Mitochondrial RNA synthesis can also be specifically inhibited by low concentrations of ethidium bromide and actinomycin D. Preliminary work using intact mitochondria suggests that ethidium bromide and rifampicin may also act to inhibit mitochondrial protein synthesis directly (Dube et al., 1973; Grivell and Metz, 1973; Avadhani and Rutman, 1975).

Mitochondrial tRNAs are thought to resemble bacterial tRNAs more closely than cytoplasmic tRNAs. As in bacterial systems, N-formyl-methionyl-tRNA is the initiator tRNA (Smith and Marcker, 1968). There also appear to be similarities between synthetases, since the bacterial enzymes can be used to charge mitochondrial tRNAs in vitro. This is noteworthy since the mitochondrial synthetases seem to be under nuclear control (Davey et al., 1969).

Ribosomal RNAs isolated from animal mitochondria are smaller, 12S and 16S (Borst and Grivell, 1971), than ribosomal RNAs found in either prokaryotic or eukaryotic ribosomes. Using hybridization experiments, Aloni and Attardi (1971b) showed that ribosomal RNAs isolated from HeLa mitochondria were encoded by mitochondrial DNA. The G+C content of mitochondrial rRNA is considerably lower than that of both eukaryotic and typical bacterial rRNAs, giving it a lower stability in its secondary structure (Dawid, 1973).

In mitochondria of Tetrahymena a ribosomal RNA sedimenting at 5S has been observed (Chi and Suyama, 1970). However in animals, 5S RNA appears to be absent. A ribosomal RNA sedimenting at 3S was isolated from mitochondria of hamsters by Dubin et al. (1974). Even though the 3S RNA did not associate with the mitochondrial large subribosomal particles, the authors postulated that it may correspond to the 5S RNAs found in other ribosomes.

The Problem

When this study was initiated, little had been reported concerning functional activities of mammalian mitochondrial ribosomes. It was generally assumed that, like the mitochondrial ribosomes isolated from fungi, mammalian mitochondrial ribosomes were indistinguishable from bacterial ribosomes in terms of function. Initial studies were complicated by the very low yield of mitochondrial ribosomes, their relatively low activities in the assays used, and the difficulty of obtaining mitochondrial ribosomes free of cytoplasmic contaminants.

The unexpected physical-chemical properties of the mammalian mitochondrial ribosomes and the possibility that phylogenetic differences existed between protein synthesis mechanisms within mitochondria of lower and higher eukaryotes led me to examine some functional activities of the isolated 55S ribosomes.

This research was directed towards determining the role of mitochondrial ribosomes in protein synthesis. To this end I decided to look at one specific but crucial reaction, that of peptide bond formation on mitochondrial ribosomes. First, the peptidyl transferase activity was localized on the mitochondrial ribosome. Specific antibiotic probes were used to characterize this site with respect to the analogous site on other ribosomes. The antibiotics chosen were those known to act on the peptidyl transferase site of prokaryotic ribosomes. Some were known to inhibit protein synthesis directly within mitochondria (for example chloramphenicol), whereas the effect of others (for example carbomycin and lincomycin) was open to interpretation. In my studies I describe the sensitivities of isolated mammalian mitochondrial ribosomes with respect to these drugs.

Under normal conditions of protein synthesis, formation of peptide bonds by peptidyl transferase is promoted by complex interactions occurring among the two ribosomal subunits, mRNA, elongation factors and tRNA. There are two substrate binding sites on the ribosome:

an "A" site for the binding of amino acyl-tRNA and a "P" site for the binding of peptidyl-tRNA. Binding of substrate to the "A" site of ribosomes occurs readily and specifically. The binding of peptidyl-tRNA to the "P" site is weak in the absence of stabilizing interactions among the various components of the functioning ribosome complex. In the presence of alcohol (methanol or ethanol) the binding of peptidyl-tRNA to the "P" site on the large subunit is promoted in the absence of the many otherwise required components. Alcohol can also stimulate the binding of a N-acetyl-aminoacyl-CCA fragment, produced by RNase T_1 digestion of N-acetyl-aminoacyl-tRNA, to the "P" site. With the two sites occupied, peptidyl transferase can catalyze formation of a peptide bond. The mechanism of action in the presence of ethanol is thought to be the same as that occuring *in vivo* in protein synthesis (Monro *et al.*, 1968). Furthermore, the sensitivity to specific antibiotic inhibitors is retained (Monro *et al.*, 1968).

The "fragment reaction" of Monro (1967), which measures peptide bond formation between puromycin and the N-acetyl-aminoacyl-tRNA fragment, takes advantage of this alcoholic medium in order to examine the fine structure of the peptidyl transferase locus. The large subribosomal particle containing the intact peptidyl transferase locus is the minimal ribosomal structure required. Using this assay it was established that energy and soluble factors

are not required for the formation of peptide bonds (Monro et al., 1969).

In addition to the predominant peptide bond forming reaction occurring on ribosomes in the presence of alcohol, the peptidyl transferase also catalyzes the transesterification of the amino acid (Scolnick et al., 1970). The rate of this reaction is one order of magnitude smaller than that of the peptide bond forming reaction and it appears to be dependent on the nature of the substrate bound to the "A" site. The peptide bond forming reaction is favored when the substrate binding to the "A" site is either an amino acylated tRNA or puromycin, whereas the transesterification reaction will occur if the substrate is an uncharged tRNA (Scolnick et al., 1970). Hydrolysis of the amino acyl-tRNA occurring at room temperature during the course of the reaction, therefore, will influence the formation of the ester product. However, the treatment of the reaction mixture with alkali (see "methods," Miskin et al., 1970) eliminates the ester without affecting the N-acetyl[^{3}H]leucyl-puromycin product. The radioactive product extracted into ethyl acetate under these conditions measures only the peptide bond forming activity of the peptidyl transferase.

The fragment reaction is particularly well suited for studies correlating structure and function of ribosomes because it requires a minimal ribosomal structure and a limited number of intermediate steps immediately

surrounding the peptidyl transferase activity. The peptidyl transferase center of ribosomes has been analyzed extensively by the use of antibiotic probes, i.e. inhibitors which bind specifically to proteins in or around the active center (reviewed in Pestka, 1971; Vazquez, 1974). These studies have yielded information concerning the structure-function relationship of ribosomal proteins in the locus. For example, some inhibitors may affect the binding of aminoacyl-tRNA to the "A" site whereas others will affect the binding of peptidyl-tRNA to the "P" site and still others may inhibit the peptidyl transferase itself. Antibiotics specific for the peptidyl transferase center of prokaryotic ribosomes were chosen for this study because of the prior observation that protein synthesis in intact mitochondria resembles that of bacteria.

Chloramphenicol, which specifically inhibits the peptidyl transferase activity of prokaryotic ribosomes (Pestka, 1971), is one of a few classical antibiotics used to discriminate the protein synthesizing systems of prokaryotic and eukaryotic organisms. On the basis of its chloramphenicol sensitivity, mitochondrial protein synthesis was determined to be of the bacterial type (Rendi, 1959; Clark-Walker and Linnane, 1966; de Vries et al., 1971; Kroon and de Vries, 1971). The early studies, however, were done with intact mitochondria. Investigators were not able to rule out a direct inhibitory

effect of chloramphenicol on oxidative phosphorylation with consequently indirect effects on protein synthesis. Using isolated mitochondrial ribosomes from rat livers, de Vries et al. (1971) showed a direct effect of chloramphenicol on their peptidyl transferase activity. The susceptibility of bovine liver mitochondrial ribosomes to chloramphenicol has been examined and compared to a standard inhibition profile obtained for E. coli ribosomes.

Members of the streptogramin group of antibiotics have been shown to interact directly with the 50S subunit of prokaryotic ribosomes by inhibiting the binding of the amino acyl end of amino acyl-tRNA to the "A" site (Ennis, 1966; Mao and Putterman, 1969; Vazquez, 1966b). Furthermore, mikamycin, a member of this group, has a direct effect on protein synthesis occurring within intact yeast mitochondria (Towers et al., 1972). The susceptibility of bovine mitochondrial ribosomes to PA114A and vernamycin A was investigated in this study.

Considerable controversy exists in the literature concerning the action of the lincosamines and macrolides within mitochondria of higher organisms (Towers et al., 1972; Firkin and Linnane, 1969; Kroon, 1969; Williams and Birt, 1972). Unlike mitochondria from fungi, the intact mammalian mitochondria respond differentially to these antibiotics. For example, protein synthesis within intact organelles is resistant to erythromycin (Firkin and Linnane, 1969; Kroon, 1969) but highly sensitive to the

action of carbomycin and spiramycin (Towers et al., 1972), all members of the macrolide group. The intact organelles are also insensitive to lincomycin (Kroon and de Vries, 1971). A permeability barrier for erythromycin and lincomycin at the level of the mitochondrial membrane has been observed in mammalian mitochondria (Kroon and de Vries, 1971). This conclusion came as a result of experiments in which mitochondria were ruptured by sonication or by treatment in a hypotonic medium. Protein synthesis was then sensitive to the action of these drugs (Kroon and de Vries, 1971). The observed high sensitivity to carbomycin and spiramycin may be due to a 30-fold concentration (de Vries et al., 1973) of these drugs within intact organelles. Studies with isolated mammalian mitochondrial ribosomes were necessary to determine whether mitochondrial ribosomes are indeed susceptible to these antibiotics.

Poly U dependent polyphenylalanine synthesizing systems have been used as model reactions for other ribosomes by many investigators. These systems are not as restrictive as the in vivo system since initiation factors and initiator tRNA are not required. Nevertheless, the assays depend on functional and complete ribosomes. Both ribosomal subunits are required as well as elongation factors, EF-T and EF-G, phenylalanyl-tRNA, energy in the form of GTP and ATP, and the correct ionic conditions. In my studies, a poly U-directed system developed by

Hosokawa et al. (1966) was used to ascertain the functional integrity of beef liver mitochondrial ribosomes. It was, furthermore, a convenient assay for determining whether bacterial elongation factors and tRNA can support the mitochondrial ribosomal activity.

A partial reaction of protein synthesis can be used to examine the GTP binding ability of ribosomes. The binding and subsequent hydrolysis of GTP is necessary for three different steps in protein synthesis: (a) binding of the initiator tRNA to the peptidyl site mediated by the initiation factor IF-2; (b) binding of all incoming aa-tRNAs to the amino acyl site mediated by the elongation factor EF-T; and (c) translocation of the peptidyl-tRNA from the "A" site to the "P" site mediated by the elongation factor EF-G. A large body of evidence suggests that there is a common binding site on the large subunit for IF-2, EF-T and EF-G (Hamel et al., 1972; Hamel and Nakamoto, 1972; Highland et al., 1971; Richman and Bodley, 1972) and that this site is composed mainly of the r-proteins L_7 and L_{12} (Highland et al., 1974; Highland et al., 1973). I examined mitochondrial ribosomes for their ability to bind [^{3}H]GTP as an additional measure of their competence in the partial reactions of protein synthesis.

CHAPTER 2
MATERIALS AND METHODS

Materials

Chloramphenicol was obtained from Sigma Chemical Company. Lincomycin-HCl (U-19149A, lot XS-610) and Celesticetin (U-4819, lot 16376) were the generous gifts of Dr. George B. Whitfield from Upjohn and Company. PA114A (lot 1454-172A) and Carbomycin (lot 42464) were the generous gifts of Dr. Nathan Belcher from Pfizer Company. Tylosin tartrate (lot 4 PE77) was donated by Dr. Robert Hosley from the Lilly Research Laboratories. Vernamycin A (SQ16515, Batch 5A) was given by Miss Barbara Stearns from Squibb. These antibiotics are prepared by the biomedical research groups in each company and are distributed in small quantities (5-100 mg) to interested investigators. They do not appear to lose activity upon storage for 3 to 4 years and no impurities affecting their activity have been reported. Puromycin diHCl was purchased from Nutritional Biochemical Company, and E. coli de-aminoacylated tRNA (stripped tRNA) from General Biochemicals. [4,5-^{3}H]L-leucine, 55 Ci/mmole; [^{3}H]L-phenylalanine, 7 Ci/mmole; and [8-^{3}H]GTP tetrasodium, packed in 30% ETOH, 12 Ci/mmole were obtained from Schwarz-Mann. Permablend, containing 91% PPO and 9% Dimethyl POPOP, was purchased from Packard.

Methods

Preparation of Mitochondria

Bovine livers of freshly killed animals were obtained at the slaughterhouse. Alternatively, rat livers were obtained from young Sprague-Dawley rats, fasted for 18 hr and killed by decapitation. The livers were cooled promptly by placing them in ice. All preparative procedures were carried out at 4° C.

The livers were sliced, ground and homogenized in either a potter Elvehjen glass homogenizer or a Super Dispax flow-through homogenizer, Model SD-45K, Tekmar Co., in 6 volumes of isolation medium A (0.34 M sucrose, 5 mM Tris-HCl, pH 7.4) or isolation medium B (0.25 M sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.6). Whole cells and cell debris were sedimented by centrifugation at 970 x g for 10 min in a Sorvall GSA rotor. Mitochondria were resuspended three times in isolation medium and sedimented by differential centrifugation for 10 min at 5200 x g in the GSA rotor. This procedure has been described previously by O'Brien (1971).

Preparation of Mitochondrial Ribosomes

During the course of this study the method of obtaining ribosomes from mitochondria was varied. Two procedures are described. The second procedure reduces the contamination of mitochondrial ribosomes by cytoplasmic 80S ribosomes.

Procedure 1

Three-times-washed mitochondria were resuspended at 20 mg protein/ml standard buffer (20 mM $MgCl_2$, 100 mM KCl; 20 mM triethanolamine, pH 7.6, 5 mM 2-mercaptoethanol). Heparin and oligonucleotides, prepared by partial digestion of yeast tRNA with NaOH (Spencer and Poole, 1965), were added to 0.05 mg/ml and 0.2 mg/ml respectively in order to diminish the ribonuclease degradation of mitochondrial ribosomes. Mitochondria were lysed by the addition of Triton X-100 to 2% and DOC to 0.5%. This lysate was centrifuged for 10 min at 60,000 x g in a Spinco type 30 rotor to remove membrane fragments. Crude mitochondrial ribosomes were obtained from this supernatant by centrifuging through a 2 ml layer of standard T buffer containing 24% sucrose for 3 hours at 230,000 x g in a Spinco type 65 rotor.

The pellets were rinsed gently with T buffer and finally resuspended in a small volume of the same buffer containing 50 µg/ml heparin and 550 µg/ml puromycin. The mixture was incubated for 5 min at 37° C and returned to the ice bath promptly after incubation. The ribosome solution was slightly turbid after this treatment and was clarified by centrifugation at 14,000 x g for 10 min in a Spinco type 65 rotor. The volume of the resulting supernatant was adjusted to the appropriate level with T buffer and layered directly onto 10 to 30% sucrose density gradients to purify the mitochondrial ribosomes further.

Procedure 2

In a modified version of the above procedure, three-times-washed mitochondria were treated with 0.5% digitonin (de Vries et al., 1971) in order to remove the outer mitochondrial membranes and thus diminish the contamination by cytoplasmic 80S ribosomes. Heparin and oligonucleotides were omitted from this preparation. Prior to lysis, mitochondria were resuspended in DVT buffer (10 mM Mg_2Cl; 0.1 M KCl; 5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.6; 0.1 mM EDTA) and lysed by the addition of Triton X-100 to 2%. DOC was omitted. The lysate was treated with puromycin and mitochondrial ribosomes were obtained as described for method 1.

Sucrose Density Gradients

Linear 10-30% sucrose density gradients were prepared three at a time with a Beckman density gradient former. All solutions contained RNase-free sucrose. Gradients were centrifuged for the appropriate time and speed in the Beckman SW 27 rotor at 4° C. The gradients were fractionated by pumping a 34% sucrose solution into the bottom of the centrifuge tube thereby displacing the solution at the top. The Gilford model 2400 spectrophotometer was used to monitor the gradients at 260 nm as they percolated through a modified Gilford flow-through cell. E. coli ribosomes and subribosomal particles, analyzed on separate gradients, were used as sedimentation coefficient standards.

Fractions of 0.5 ml volumes were collected at 20 sec intervals. Contents of tubes from each sedimenting species were pooled and concentrated by centrifugation for 3 to 6 hrs at 230,000 x g in the Spinco type 65 rotor. These ribosomes were used immediately in functional studies.

Preparation of Mitochondrial Subribosomal Particles

Mitochondrial subribosomal particles can be obtained in two ways. "Native" subunits are by-products of the monosome preparation, obtained directly from the sucrose density gradients in T buffer. "Derived" subunits are prepared by dissociating 55S monosomes in suitable buffers. Both "native" and "derived" particles were sometimes used directly in functional studies. Alternatively, they were washed with buffers of varying Mg^{2+}/KCl ratios known to remove defined groups of ribosomal proteins (David Matthews, personal communication) prior to being studied. Ribosome cores thus produced can be separated from the released proteins by sucrose density centrifugation or by sedimentation through a 4 ml layer of the same wash buffer containing 10% sucrose. Sucrose density gradient centrifugation proved to be the more useful and reproducible method.

The wash buffers used contained 20 mM triethanolamine, pH 7.5, 5 mM 2-mercaptoethanol and varying quantities of $MgCl_2$ and KCl,

Buffer S: 10 mM $MgCl_2$, 0.1 M KCl;

Buffer BTR: 10 mM $MgCl_2$, 0.5 M KCl;

Buffer O: 5 mM $MgCl_2$, 0.3 M KCl;

Buffer Z: 5 mM $MgCl_2$, 0.5 M KCl;

Buffer Y: 5 mM $MgCl_2$, 1 M KCl;

Buffer A: 1 mM $MgCl_2$, 1 M KCl.

Preparation of Bovine Liver 80S Cytoplasmic Ribosomes

Bovine liver cytoplasmic 80S ribosomes were obtained as by-products of mitochondrial ribosome preparations when "procedure 1" was employed. They were subjected to the same preparative manipulations as mitochondrial ribosomes and thus served as useful internal controls.

Alternatively, 80S cytoplasmic ribosomes were prepared directly from the microsomal fraction of the beef liver post-mitochondrial supernatant. Microsomes were resuspended in buffer I (20 mM triethanolamine, pH 7.5; 5 mM $MgCl_2$, 0.1 M KCl; and 5 mM 2-mercaptoethanol) and lysed with 2% Triton X-100 in order to release their ribosomes. Membranous material was removed by centrifugation at 60,000 x g in a Spinco type 30 rotor for 10 min.

Ribosomes were removed from the resulting supernatant by centrifuging them through 1/4 volume of the same buffer containing 34.5% sucrose for 12 hrs at 63,000 x g in a Spinco type 60 rotor. Subsequently, the cytoplasmic ribosomes were incubated with puromycin (550 µg/ml) for 5 min at 37° C to free the nascent proteins

from the ribosomes. Following incubation the mixture was cooled quickly on ice. The sample was layered onto 10-30% sucrose density gradients prepared as described. Gradients were centrifuged for the appropriate time in a Beckman SW 27 rotor. After fractionation the material corresponding to the 80S peak was pooled. Ribosomes were concentrated by centrifugation and used directly in activity studies.

Isolation of Crude Mitochondrial Factors

Bovine or rat liver mitochondria were resuspended in two volumes of KTM (40 mM KCl; 20 mM Tris-HCl, pH 7.5; 15 mM $MgCl_2$; 6 mM 2-mercaptoethanol) according to the method of Leister and Dawid (1974). The organelles were ruptured by sonication in a Bronwill Biosonik 2 Sonicator using the small probe (4 mm diameter) at maximum intensity of 150 watts. Four 15 sec bursts were given at 20 sec intervals. The lysed mixture was centrifuged at 14,000 x g for 10 min in a Spinco type 65 rotor to remove membranous material. The supernatant was adjusted to 0.5 M KCl and centrifuged once more at 230,000 x g for 4 hrs in a Spinco type 65 rotor. The upper 3/4 of the resulting supernatant was removed and concentrated 20-fold with dry Sephadex G-25, 3 g/10 ml of supernatant (Leister and Dawid, 1974). Dry Sephadex G-25 was added to the supernatant and was allowed to swell. This mixture was placed within the barrels of 5 ml syringes containing nylon net

and luer lok fittings over their narrow openings. The syringe assemblies were placed within centrifuge tubes and centrifugation was carried out at 1/2 maximum velocity in an IEC desk top centrifuge for 2 min at 4° C. The concentrated crude factors were collected in the centrifuge tubes. Subsequently, the sample was passed through a column of Sephadex G-25. The void volume, containing the factors, was collected and used immediately in poly U-dependent polymerization of phenylalanine assays.

Preparation of E. coli Ribosomes and Crude Factors

The procedure of Nirenberg (1963) was used to prepare E. coli ribosomes and crude factors. E. coli K-12, strain Hfr DIO RNase$^-$ or strain 1200 F$^-$end A 1100 rnsA Su$^-$, cells were grown to early exponential phase in nutrient broth at 37° C. After harvesting in the cold, the cells were resuspended in ice cold standard buffer (10 mM Tris-HCl, pH 7.8; 60 mM KCl, 14 mM $Mg(OAc)_2$; 6 mM 2-mercaptoethanol). They were ruptured by sonication in a Bronwill Biosonik Sonicator using the small probe at maximum intensity. Four 15 sec bursts were given at 20 sec intervals. Cell debris was removed by centrifugation in a Spinco type 65 rotor for 10 min at 14,000 x g. The resulting supernatant was centrifuged at 230,000 x g for 2 hr in the Spinco type 65 rotor in order to sediment all the ribosomes. The upper 2/3 of the new supernatant was removed and passed through a Sephadex G-50 column.

The void volume, containing the factors, was collected and stored frozen in small aliquots at -70° C. The ribosome pellets were washed either in standard buffer or in a high salt buffer W (10 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 5 mM 2-mercaptoethanol; 1 M NH_4Cl). Ribosomes prepared in this manner were resuspended in the buffer of choice and stored frozen at -70° C.

Preparation of N-acetyl[^{3}H]leucyl-tRNA

Crude amino acyl tRNA synthetase was prepared from E. coli by the method of Nishizuka et al. (1968). Exponential phase E. coli cells were resuspended into 10-15 ml of cold buffer B (10 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 2 mM 2-mercaptoethanol) and lysed by sonication as described. Cell debris was removed by centrifugation at 14,000 x g for 10 min in the Spinco type 65 rotor. The top third of the 230,000 x g supernatant obtained by centrifugation for 2 hr in the Spinco type 65 rotor was dialyzed overnight in 100 volumes of a buffer containing 10 mM Tris-HCl, pH 7.4 and 2 mM 2-mercaptoethanol. Aliquots were stored frozen at -70° C in a Revco freezer.

Stripped E. $coli_B$ tRNA was charged with [^{3}H]leucine by the method of Nishizuka et al. (1968). The reaction mixture contained: 100 mM Tris-HCl, pH 7.2; 25 mM $MgCl_2$; 4 mM ATP; 10 mM glutathione; 10 mg stripped tRNA; 0.80 mg protein crude synthetase; 0.5 mCi[4,5-^{3}H]leucine, 55 Ci/mmole in a volume of 10 ml. The reaction was started

by the addition of synthetase. Incubation was for 30 min at 37° C. The reaction was stopped by the addition of an equal volume of glass distilled phenol. The phenol extraction was performed to remove all protein and thereby purify the leucyl-tRNA. The tRNA fraction was precipitated from the aqueous phase, adjusted to 2% KOAc, with two volumes of cold ethanol. It was washed 2 times with cold 70% ethanol, and once with ether to remove all traces of phenol. After drying at 0° C under vacuum, the sample was resuspended into a small volume of water.

[^{3}H]leucyl-tRNA was acetylated by the method of Haenni and Chapeville (1966). [^{3}H]leucyl-tRNA was dissolved in a small volume of 0.2 sodium acetate buffer, pH 5.0. Three 40 µl aliquots of acetic anhydride were added at 40 min intervals to the mixture maintained at 0° C. The product, acetyl-[^{3}H]leucyl-tRNA, was precipitated with cold ethanol, washed several times and dried under vacuum. It was then resuspended in a small volume of H_2O and stored frozen at -70° C. This method of acetylation is 100% efficient (Haenni and Chapeville, 1966).

Peptidyl Transferase Assay

The peptidyl transferase activity of ribosomes was measured by the "fragment reaction" of Monro (1971) with a few modifications. N-Acetyl[^{3}H]leucyl-tRNA (de Vries et al., 1971) was reacted instead of a leucyl-CCA terminal fragment obtained by treatment of the molecule with RNase T_1

(Monro et al., 1968). The reaction mixture contained: 36 mM Tris-HCl, pH 7.5; 267 mM KCl; 36 mM $Mg(OAc)_2$; 0.66 mM puromycin; 1 A_{260} units of ribosomes; 83 nM Ac[^{3}H]leu-tRNA (6,000 to 10,000 cpm) and 33% v/v ethanol in a volume of 0.15 ml. The reactions were incubated at 25° C and were terminated by the addition of KOH to a concentration of 0.6 N. The mixtures were then incubated at 40° C for 3 min to break down any [^{3}H]leucyl ethyl ester by-products, and then were neutralized by the addition of 1 ml 1 M sodium phosphate, pH 7.0 (Miskin et al., 1970). The N-acetyl[^{3}H]leucyl-puromycin product was extracted into 1.5 ml ethyl acetate by shaking the reaction tubes for 10 sec on a vortex mixer and centrifuging in a desk top centrifuge at low speed for 1 min to separate the phases. 1 ml of the ethyl acetate extract was mixed with 10 ml triton-toluene-permablend (50% triton, 0.5% PPO and 0.05% POPOP) liquid scintillation mix and counted as above. Counting efficiencies were 25%.

Poly U-Dependent Polymerization of [^{3}H]Phenylalanine

Ribosomal activities were determined using the poly U-dependent protein synthesizing system of Hosokawa et al. (1966). The reaction mixture contained: 10 mM Tris-HCl, pH 7.8; 50 mM KCl; 20 mM $Mg(OAc)_2$; 6 mM 2-mercaptoethanol; 1 mM DTT; 0.032 mM GTP; 5 mM potassium phosphoenol pyruvate; 1 mM ATP; 0.64 mg/ml poly U; 25 mM tyrosine; 50 mM each of 18 amino acids in a mixture

excluding tyrosine and phenylalanine; 5.4 μM [^{3}H]phenylalanine, 1.82 Ci/mmole; 0.5 mg/ml tRNA; 0.1 mg/ml pyruvate kinase (lyophilized or as an $(NH_4)_2SO_4$ suspension); 0.1 to 1.0 mg/ml crude factors; 0.5 to 2.0 A_{260} units of ribosomes in a total volume of 0.25 ml. The reaction was started by the addition of factors, except when ribosomes were preincubated in the above reaction mixture in the absence of poly U for 5 min at 30° C to allow for termination of protein synthesis on any endogenous mRNA. In these latter cases, the reaction was started by the addition of poly U. Reaction tubes were incubated at either 30° or 37° C. At specified times 50 μl aliquots were placed onto Whatman 3 MM filter papers and processed by the method of Mans and Novelli (1960). [^{3}H]phenylalanine incorporated into hot acid-insoluble protein was measured by counting the filter papers in toluene-permablend (0.5% PPO and 0.05% POPOP) liquid scintillation mix in a Beckman LS 330 liquid scintillation spectrometer. The samples were counted for 10 min each at an efficiency of 4%.

Binding of [^{3}H]GTP to Ribosomes

The millipore filtration technique of Bodley *et al.* (1970) was used to measure the binding of [^{3}H]GTP to ribosomes. The reaction mixture contained: 10 mM Tris-HCl, pH 7.4; 10 mM NH_4Cl; 20 mM $Mg(OAc)_2$; 5 mM 2-mercaptoethanol; 5 pmoles of ribosomes:* 42 pmoles [8-^{3}H]GTP

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* See Appendix A for calculations.

(0.5 μCi) in a volume of 50 μl. The reactions were started by the addition of [^{3}H]GTP. Following incubation for 5 min at 0° C, the reactions were terminated by the addition of 3 ml cold buffer containing 10 mM Tris-HCl, pH 7.4; 10 mM $Mg(OAc)_2$; 10 mM NH_4Cl. This mixture was immediately filtered through a millipore filter and washed 3 times with 3 ml aliquots of the same buffer. This washing procedure was done very quickly as emphasized by Bodley et al. (1970) to minimize disaggregation of the metastable complex. The filters were then air dried, overlaid with toluene-permablend liquid scintillation mix and counted as above. The efficiency of counting was 16%.

Efficiency of Counting

To correct for self-absorption and quenching mock reaction mixtures for each assay condition were prepared and counted directly in the usual scintillation cocktail mix. For the poly U directed assay, this involved pipetting 50 μl samples containing 5.49×10^6 dpm of [^{3}H] phenylalanine, 1.85 Ci/mmole directly onto Whatman 3 MM filter paper discs. Increasing quantities of E. coli and mitochondrial factors (0-100 μl) at concentrations normally used in the reactions were layered on top. For the [^{3}H]GTP binding reaction, 10 μl of [^{3}H]GTP containing 1.1×16^6 dpm were placed on a millipore filter and overlaid with 5 to 10 μl of E. coli ribosomes whose concentrations equaled those used in the reactions. The filters were

air dried before counting in toluene-permablend (0.5% PPO and 0.05% POPOP).

And, finally, the [^{3}H] efficiency for the "fragment reaction" was determined by adding 10 µl (8.804 mg) of a standard [^{3}H] toluene solution containing 2.6×10^6 dpm/g directly to 10 ml triton-toluene-permablend (50% triton, 0.5% PPO and 0.05% POPOP). One ml of ethyl acetate was added as well to control for quenching due to this solvent and the sample was counted.

Counting efficiencies for each assay were determined by dividing the cpm experimentally obtained by the dpm added and multiplying by 100.

CHAPTER 3
RESULTS

Preparation of Mitochondrial Ribosomes

Mitochondria are studded with cytoplasmic ribosomes *in vivo*. To eliminate a large portion of the cytoplasmic contaminants, the mitochondria were washed several times in isolation medium. Two procedures were used to prepare mitochondrial ribosomes and subribosomal particles as described in "methods." In initial studies (procedure 1) the mitochondria were not treated with digitonin and therefore contained intact outer membranes. Lysis of mitochondria with Triton X-100 released not only mitochondrial ribosomes, but 80S cytoplasmic ribosomes as well. Mitochondrial 55S ribosomes were readily separated from 80S cytoplasmic ribosomes by centrifugation in sucrose density gradients (Figure 1). However, there was a small but variable (5-15%) contamination of 55S ribosomes by 60S cytoplasmic subribosomal particles. This contamination was analyzed by centrifuging pooled 55S ribosomes in sucrose density gradients under dissociating conditions (Figure 2). Under these conditions, the derived mitochondrial subribosomal particles can be distinguished from the residual 60S cytoplasmic subribosomal particles also on the basis of characteristic differences in buoyant density (O'Brien *et al.*, 1974).

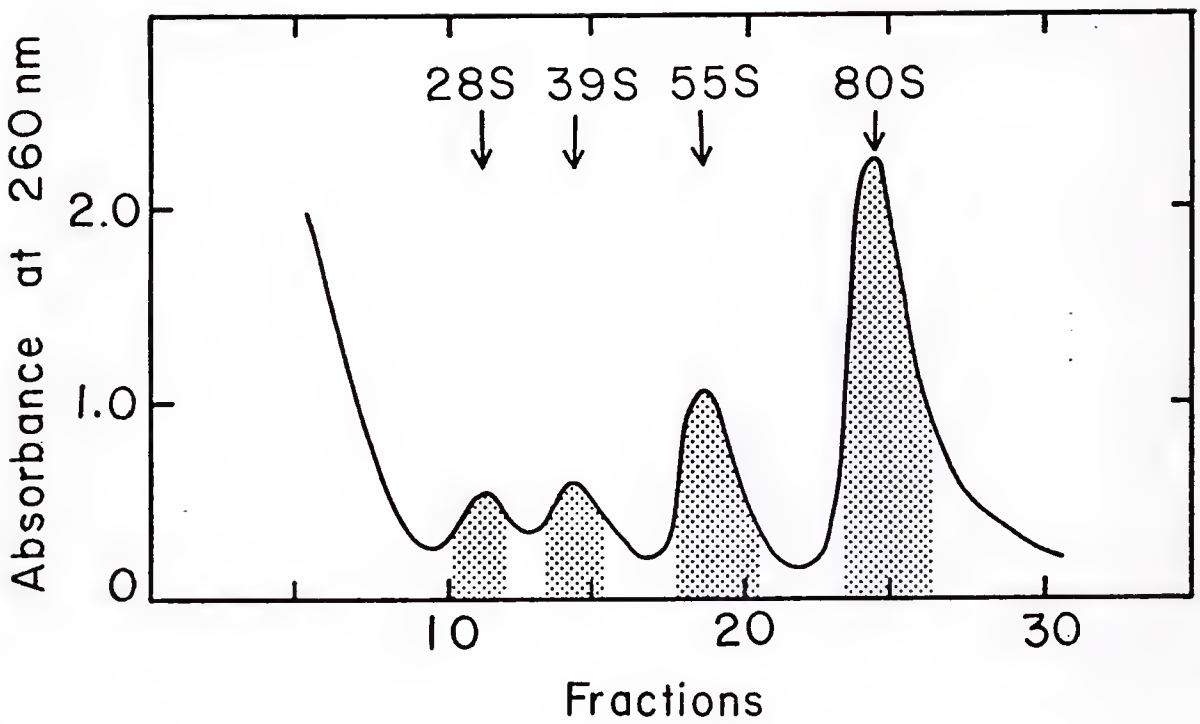


Figure 1. Sucrose density gradient centrifugation of crude ribosomes isolated from bovine liver mitochondria (1.1 g of protein) by procedure 1 as described under "methods." The crude ribosomal fraction was layered onto linear 10-30% sucrose gradients in buffer T (20 mM $MgCl_2$, 0.1 M KCl, 5 mM 2-mercaptoethanol, and 20 mM triethanolamine, pH 7.5). Centrifugation was for 5 hr at 27,000 rpm in the Beckman SW 27 rotor. The shaded areas represent the fractions which were pooled.

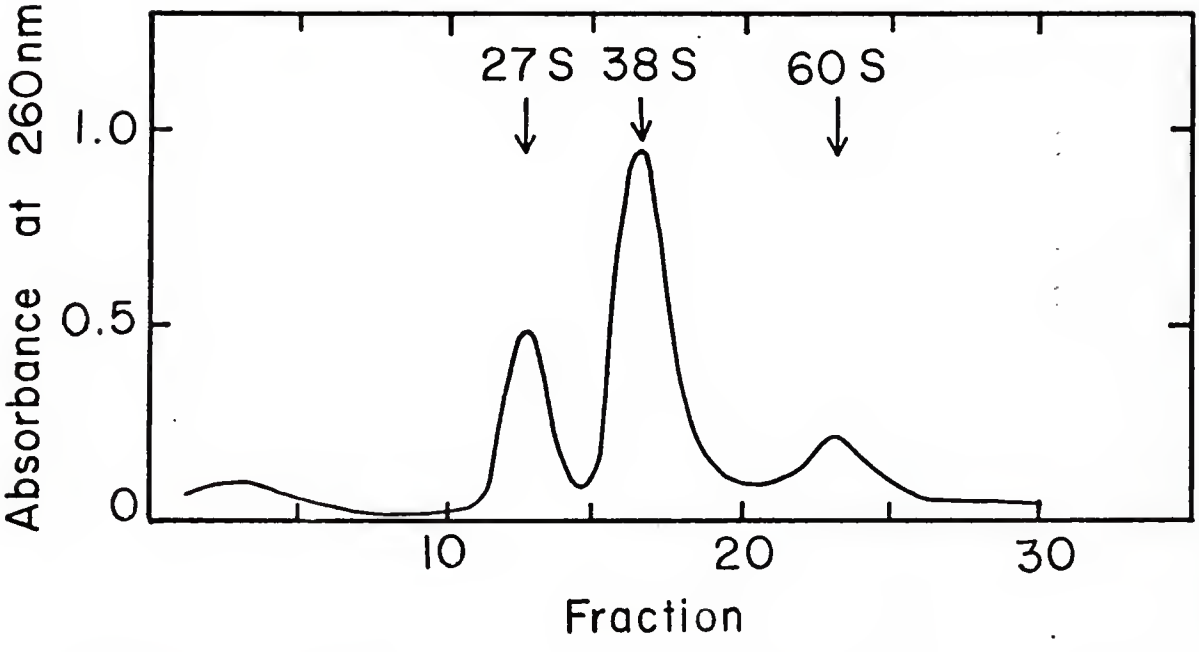


Figure 2. Sucrose density gradient analysis of isolated 55S ribosomes prepared by sucrose density centrifugation (see Figure 1). The 55S ribosomes were dissociated in buffer Z (5 mM $MgCl_2$, 0.5 M KCl, 5 mM 2-mercaptoethanol and 20 mM triethanolamine, pH 7.5) and layered onto linear 10-30% sucrose density gradients in buffer Z. Centrifugation was for 13.5 hr at 20,000 rpm in the SW 27 rotor.

In later experiments procedure 2 was used to isolate mitochondrial ribosomes. As described in "methods," mitochondria were washed with digitonin to remove the outer membranes. This procedure has the additional advantage of removing lysosomes from the mitochondrial fraction.

Figure 3 illustrates a typical sucrose density profile obtained for mitochondrial ribosomes by this method. As can be observed the peak of contaminating 80S cytoplasmic ribosomes was greatly diminished by this procedure. Mitochondrial ribosomes prepared by both methods were equally active in the assays and resulted in identical inhibition patterns with antibiotics specific for the peptidyl transferase locus.

Peptidyl Transferase Center of the Mitochondrial Ribosome: Activity

The peptidyl transferase activity of mitochondrial ribosomes was examined independently of other ribosomal activities by a modified "fragment reaction" (de Vries et al., 1971; Monro, 1971; Miskin et al., 1970) as described in "methods." The time course for peptidyl transferase activity of mitochondrial monosomes and subribosomal particles is compared to that of E. coli 70S ribosomes in the experiment depicted in Figure 4. Mitochondrial 55S ribosomes appear to be as active as E. coli 70S ribosomes. In fact, mitochondrial 55S activity was not significantly different from that of 70S ribosomes as determined by

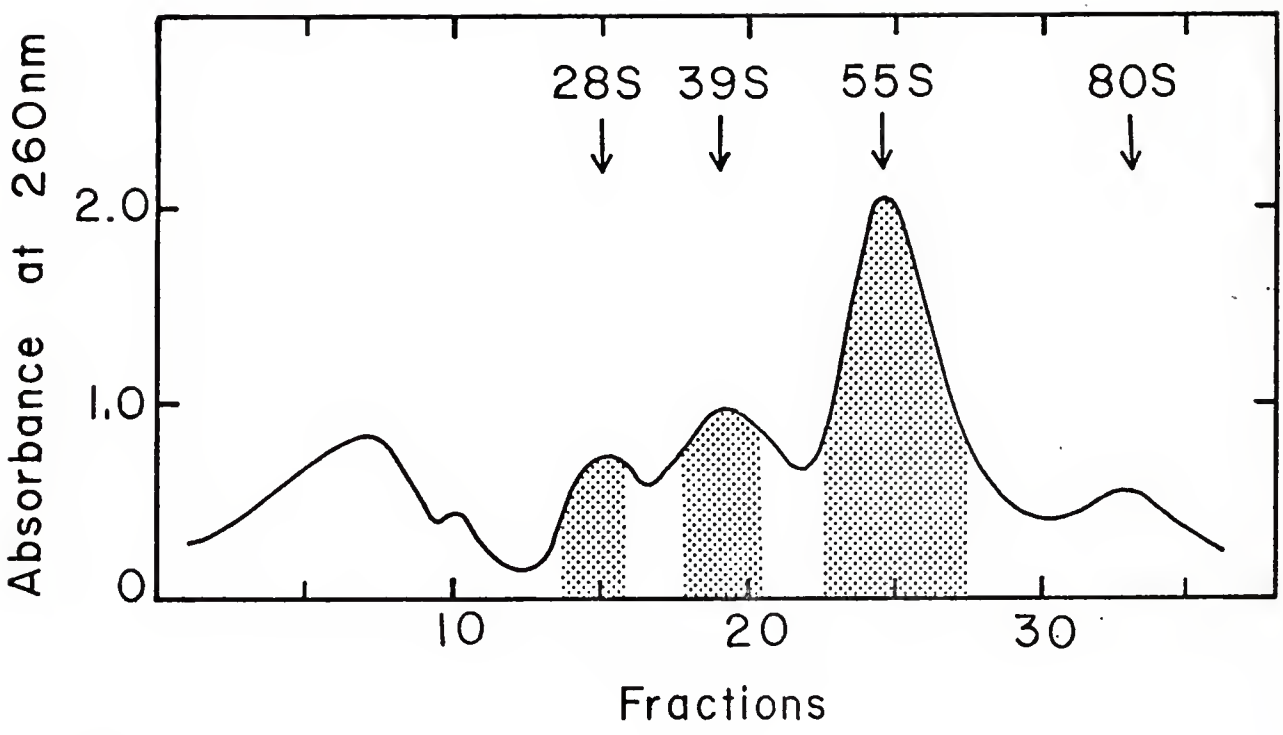
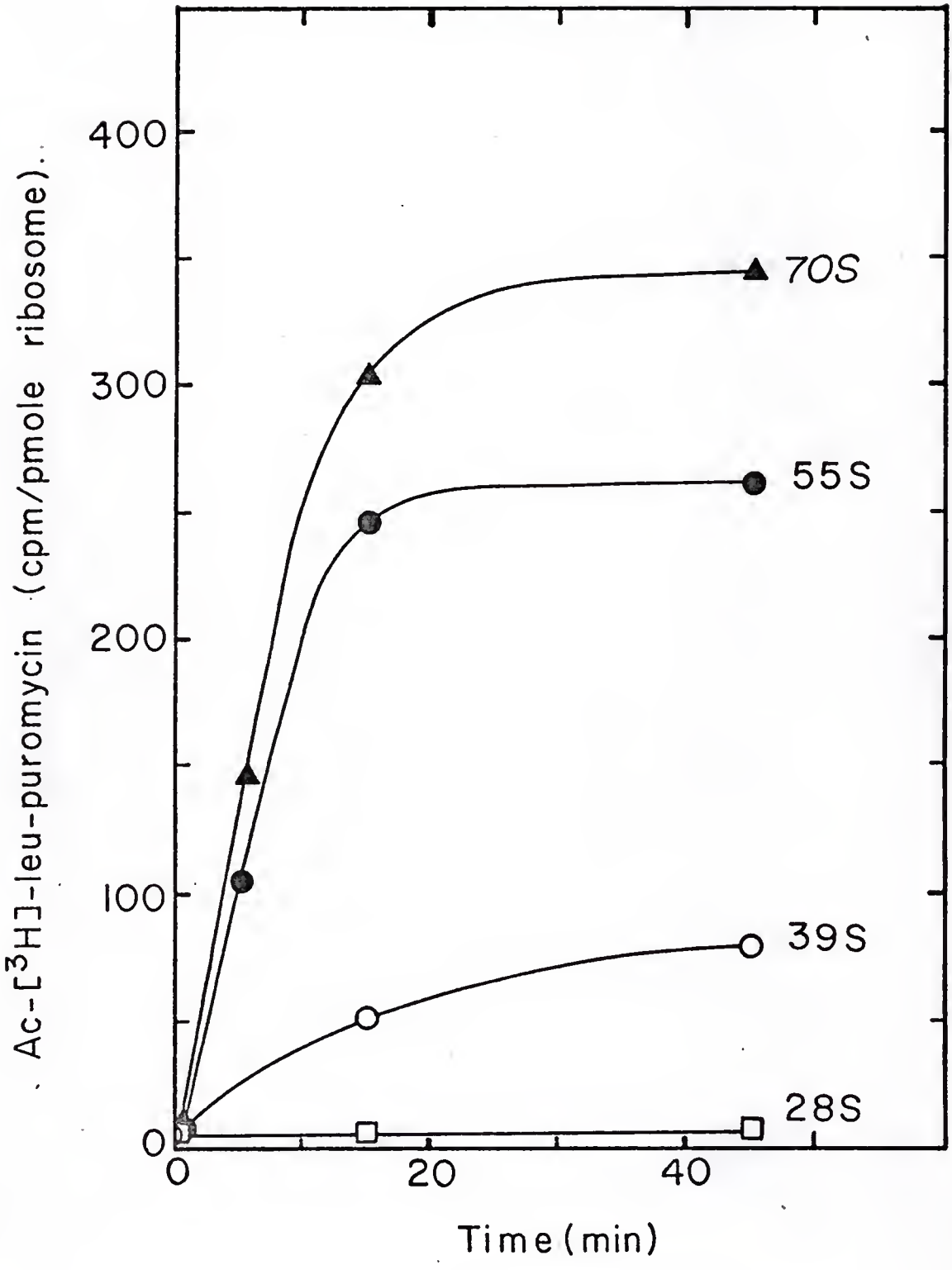


Figure 3. Sucrose density gradient centrifugation of crude ribosomes isolated from bovine liver mitochondria (4.2 g of protein) by procedure 2 as described under "methods." The crude ribosomal fraction was layered onto linear 10-30% sucrose gradients in buffer DVT (10 mM $MgCl_2$, 0.1 M KCl, 5 mM 2-mercaptoethanol, 0.1 mM EDTA and 10 mM Tris-HCl, pH 7.6). Centrifugation was for 15 hr at 20,000 rpm in the Beckman SW 27 rotor. The shaded areas represent the pooled fractions.

Figure 4. Time course of the peptidyl transferase reaction. The reaction conditions are described in "methods." Assay mixtures contained 9,245 cpm of N-acetyl[^{3}H]leucyl-tRNA and ribosomes as indicated: ▲——▲, 1.32 A_{260} units of *E. coli* 70S ribosomes (30 pmoles); ●——●, 1.09 A_{260} units of mitochondrial 55S ribosomes (35 pmoles); o——o, 1.23 A_{260} units of mitochondrial 39S subribosomal particles (68 pmoles); or □——□, 1.03 A_{260} units of mitochondrial 28S subribosomal particles (87 pmoles). Each reaction mixture was incubated at 22^0 for the prescribed time and terminated by the addition of KOH to 0.63 N as described in "methods."


Ac-[3H]-leu-puromycin (cpm/pmole ribosome)
400
300
200
100
0
0
20
40
Time (min)
70S
55S
39S
28S

t-statistic analysis of data from several experiments (Table 1).

The generally lower activities observed in these studies for cytoplasmic ribosomes have also been reported by other investigators (Neth et al., 1970; Thompson and Moldave, 1974). The specific activity of the 60S particles is only 10 to 15% that of 55S mitochondrial ribosomes. Having lower specific activities for cytoplasmic ribosomes worked to my advantage since early preparations of mitochondrial 55S ribosomes were slightly contaminated (5 to 15%) by 60S monosomes. It was calculated that only 2 to 3% of the observed activity for 55S mitochondrial ribosomes could be attributed to the 60S cytoplasmic ribosomes (see Appendix A for calculations).

To localize the peptidyl transferase activity on one of the mitochondrial ribosomal subunits, 28S and 39S particles were examined in the fragment reaction. Each subunit was free of any contamination by the other subunit or monosomes. In this manner we found the activity confined to the large 39S subunit (Table 1 and Figure 4). The 28S subribosomal particles were completely inactive. This enzymatic activity is located analogously on the large subunits of both prokaryotic and eukaryotic ribosomes (Thompson and Moldave, 1974; Neth et al., 1970; Monro, 1971). Generally, native 39S subribosomal particles were only 20% as active as the 55S monoribosomes. By comparison, the activity of the E. coli large subunit is

TABLE 1

ACTIVITIES OF VARIOUS RIBOSOME CLASSES
IN THE PEPTIDYL TRANSFERASE REACTION

Ribosome	Ac[^{3}H]Leu-puromycin cpm/pmole ribosome	SD	n
Mit 55S	143	32	6
39S	28	15	11
28S	1	-	1
Cyt 80S	7	3	6
60S	10	-	1
E. coli 70S	159	76	21
50S	148	53	3

The reaction conditions are described in "methods." The reaction vessels were incubated for 10 min. Assay mixtures contained 9,245 cpm of N-acetyl [^{3}H]leucyl-tRNA and ribosomes as indicated: 1.0 to 1.6 A_{260} units (23 to 37 pmoles) *E. coli* 50S; 0.5 to 1.1 A_{260} units (16 to 35 pmoles) mitochondrial 39S; 1.0 A_{260} unit (87 pmoles) mitochondrial 28S; 6 to 14 A_{260} units (102 to 238 pmoles) cytoplasmic 80S, and 6 A_{260} units (156 pmoles) cytoplasmic 60S ribosomes. The activities reported represent the mean of n experiments. Standard deviations, SD, were calculated for each series. The comparison of mitochondrial 55S activities with *E. coli* 70S activities yielded a t-statistic, with 25 degrees of freedom, equal to 0.48, which is not significantly different from zero.

93% of that shown by the monosome, and the cytoplasmic large subunit appears to be slightly more active than its monosome (Table 1). Our observations for the activities of prokaryotic and eukaryotic cytoplasmic ribosomal and subribosomal particles confirm published results (Monro, 1967; Neth et al., 1970; Thompson and Moldave, 1974). In the modified fragment reaction, the entire amino acyl-tRNA molecule is used rather than a RNase T_1 digest resulting in the terminal N-acetyl-leucyl-ACC fragment. Under these conditions in the mitochondrial system it is possible that the 28S subunit is required to lend binding stability to the complex. This would explain the low activities obtained with the isolated 39S subribosomal particles.

The stability of the peptidyl transferase locus of mitochondrial ribosomes was investigated by washing "native" subribosomal particles in buffers of varying ionic strength and $MgCl_2$ concentrations. Depending on the KCl and $MgCl_2$ concentrations used, discrete groups of ribosomal proteins can be removed from the 39S particles. The resulting "core" particles are purified by sedimentation through sucrose density gradients prior to activity determinations in the fragment reaction (Table 2).

Washing 39S particles in T buffer (20 mM triethanolamine, pH 7.6, 20 mM $MgCl_2$, 0.1 M KCl, 5 mM 2-mercaptoethanol) did not diminish their activity in the "fragment reaction," thereby indicating that the peptidyl transferase locus is an integral part of the mitochondrial

TABLE 2

PEPTIDYL TRANSFERASE ACTIVITY OF THE LARGE SUBRIBOSOMAL PARTICLES FROM BOVINE MITOCHONDRIA AND E. COLI PREPARED IN SELECTED BUFFERS

Buffer	$MgCl_2$/KCl (mM/M)	Relative Activity	
		Mit 39S	E. coli 50S
T	20/.1	100	-
S	10/.1	-	100
BTR	10/.5	71	-
O	5/.3	60	-
Z	5/.5	23	119
Y	5/1	4	-
A	1/1	0	-

Average of 2 or 3 experiments. The buffers contained 20 mM triethanolamine, pH 7.5, and 5 mM 2-mercaptoethanol in addition to the $MgCl_2$ and KCl concentrations indicated in the table. Native 39S particles obtained from sucrose density gradients in T buffer (see Figure 1) were pooled and pelleted by centrifugation. They were then resuspended in the indicated buffers, and centrifuged once more through sucrose density gradients of the same buffer composition. E. coli 50S subunits were prepared by sucrose density centrifugation of crude ribosomes in S buffer or Z buffer. The subribosomal particles (55 pmoles 39S and 40 pmoles 50S) were finally resuspended directly in the reaction mixture for assay of their peptidyl transferase activity as described in "methods."

ribosome rather than a loosely attached function. This was an expected result since mitochondrial ribosome structure is stable under these ionic conditions (O'Brien, 1971).

The activity of 39S particles in T buffer was chosen as the 100% activity standard for mitochondrial ribosomes in Table 2. As the ratio of Mg^{2+}/ionic strength of the buffers decreases, the mitochondrial subribosomal particles gradually lose peptidyl transferase activity. Buffer A (20 mM triethanolamine, pH 7.5, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 0.5 M KCl) is sufficient to reduce the peptidyl transferase activity by more than 75%, and buffer A (20 mM triethanolamine, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM $MgCl_2$, and 1 M KCl) abolishes this activity.

E. coli 50S ribosomes prepared by sucrose density gradient centrifugation in buffer S (20 mM triethanolamine, pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 0.1 M KCl) were used as controls. Buffer A has no detrimental effect on the peptidyl transferase activity of these ribosomes. It appears that the peptidyl transferase activity of mitochondrial ribosomes is easier to disrupt than that of E. coli ribosomes.

Susceptibility of Mitochondrial Ribosomal Peptidyl Transferase Activity to Antibiotic Inhibitors

Preliminary observations indicated that protein synthesis within intact mitochondria was sensitive to inhibitors of prokaryotic specificity and not to those of

eukaryotic specificity. For this reason several inhibitors which interact specifically with the peptidyl transferase locus of bacterial ribosomes were selected to investigate this locus in mitochondrial ribosomes. In general the peptidyl transferase activity of mitochondrial ribosomes could be inhibited by the antibiotics tested. For some of the drugs, μM concentrations were sufficient to inhibit the reaction by 50% whereas for others mM concentrations were required to achieve the same result.

A comparison of the mitochondrial ribosome response with that of other ribosomes was essential to determine whether the observed inhibition of peptidyl transferase was significant for all the drugs tested. The susceptibility of the peptidyl transferase activity of E. coli ribosomes in the presence of each antibiotic was set as the standard for sensitivity. The response of bovine liver cytoplasmic 80S ribosomes was the standard for resistance. The peptidyl transferase locus of mitochondrial ribosomes was characterized with respect to the action of each inhibitor.

The molar concentrations of vernamycin A, PA114A, and chloramphenicol required to inhibit the peptidyl transferase reactions of mitochondrial ribosomes and E. coli ribosomes by 50% are of the same order of magnitude (Table 3). But the response of mitochondrial ribosomes to lincomycin, celesticetin, carbomycin and tylosin is significantly different from that of E. coli ribosomes.

TABLE 3

MOLAR CONCENTRATION OF ANTIBIOTICS REQUIRED TO INHIBIT THE PEPTIDYL TRANSFERASE REACTION BY 50%

Antibiotic	Ribosomal Particle			
	E. coli 70S Washed	*E. coli* 70S Crude	Mitochondrial 39S	Mitochondrial 55S
Vernamycin A	$7x10^{-7}$	$1x10^{-7}$	$3x10^{-7}$	$6x10^{-7}$
PA114A	$9x10^{-7}$	$2x10^{-7}$	$7x10^{-7}$	$9x10^{-7}$
CAP	$4x10^{-5}$	-	$4x10^{-5}$	$1x10^{-4}$
Lincomycin	10^{-5}	-	$2x10^{-4}$	10^{-3}
Celesticetin	$2x10^{-4}$	-	-	$2x10^{-3}$
Carbomycin	10^{-6}	-	$5x10^{-4}$	$7x10^{-4}$
Tylosin	$3x10^{-5}$	-	-	10^{-2}

The molar concentrations required to inhibit the reactions of *E. coli* 70S and mitochondrial 39S and 55S ribosomes by 50% were estimated from the antibiotic inhibition profiles (Figures 5 to 9). Untreated *E. coli* and mitochondrial ribosomes served as the controls for 100% activity. The fragment reaction conditions are as in Table 1.

The mitochondrial ribosome reaction is inhibited by 50% when 10-- to 700-fold higher molar concentrations of these drugs are used. The difference between the responses of ribosomes from mitochondria and bacteria becomes more evident when one examines their inhibition profiles over a wide range of antibiotic concentrations as described below.

To emphasize the specificity of action of the inhibitors on the peptidyl transferase activity as measured by the modified fragment reaction, two antibiotics, vernamycin B and oleandomycin, that exert an inhibitory action at sites other than peptidyl transferase, were used as controls. The lack of inhibition of the peptidyl transferase reaction by these antibiotics is illustrated in Table 4. Vernamycin B is known to enhance the binding of vernamycin A to ribosomes and thus synergistically increase the inhibitory action of vernamycin A (Pestka, 1971). Oleandomycin, a member of the macrolide group, is known to inhibit translocation but not peptide bond formation. In fact, high concentrations (1 mM of oleandomycin) actually stimulate the binding of CACCA-acetyl-leucine to ribosomes by 25% (Celma et al., 1970). In accord with this observation, I found that high concentrations of oleandomycin also stimulated the peptidyl transferase activity of bacterial, mitochondrial or cytoplasmic ribosomes by 10 to 20% (Table 4).

In addition, an antibiotic gougerotin, known to affect the peptidyl transferase activity of both prokaryotic

TABLE 4

PEPTIDYL TRANSFERASE ACTIVITIES OF RIBOSOMES IN THE PRESENCE OF VERNAMYCIN B, OLEANDOMYCIN AND GOUGEROTIN

Antibiotic	Concentration (mM)	Ribosome		
		70S	55S	80S
		(% of control)		
Vernamycin B	0.2	98	112	-
Oleandomycin	1	120	117	111
Gougerotin	0.01	90	-	-
	0.1	60	96	113
	1.0	40	60	60

The peptidyl transferase activity of E. coli 70S (1.5 A_{260} units), mitochondrial 55S (1 A_{260} unit), and microsomal 80S (8 A_{260} units) was assessed by the fragment reaction as described in "methods." Untreated ribosomes served as the 100% activity controls.

and eukaryotic ribosomes was used. Mitochondrial ribosomes were found to be susceptible to the action of gougerotin as well.

Chloramphenicol

Chloramphenicol specifically inhibits the peptidyl transferase activity of prokaryotic ribosomes (Pestka, 1971). In this study it is shown that chloramphenicol also exerts a direct inhibitory action on the peptidyl transferase activity of mitochondrial ribosomes. A similar response by rat liver mitochondrial ribosomes has been shown by de Vries et al. (1971). Bovine liver mitochondrial ribosomes are 50% inhibited by 0.1 mM chloramphenicol, concentrations which are 3-fold greater than those required to inhibit bacterial ribosomes to the same extent (Table 3). The similarity of the responses obtained for mitochondrial and bacterial ribosomes becomes apparent when one examines their antibiotic susceptibility profiles (Figure 5). The profile for mitochondrial 39S particles closely resembles that for 70S ribosomes. These results may indicate that the site for chloramphenicol binding is partially obstructed by the 28S subunit in the 55S ribosome, thereby giving the appearance of a slightly reduced susceptibility.

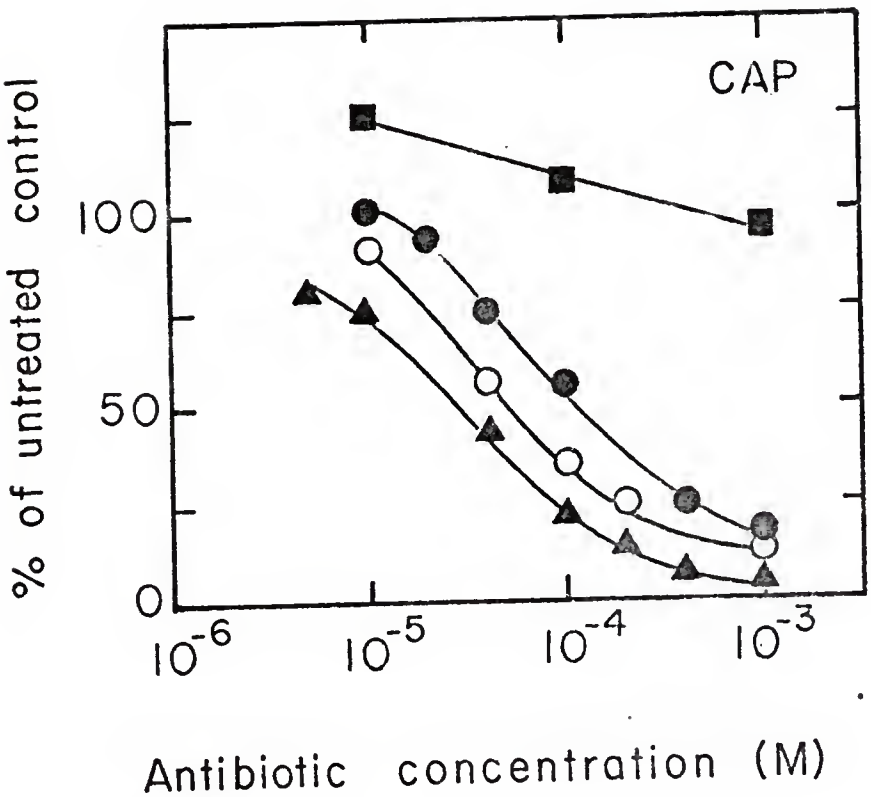


Figure 5. Effect of chloramphenicol on the peptidyl transferase activity of various ribosomes. Mitochondrial 55S, ●——●; mitochondrial 39S, o——o; E. coli 70S, ▲——▲; and microsomal 80S, ■——■, ribosomes were used. The peptidyl transferase reaction was performed as described in "methods." The reaction vessels contained 0.5 to 1.5 A_{260} units of mitochondrial or bacterial ribosomes, or 5 to 10 A_{260} units of microsomal ribosomes. The vessels were incubated for 10 min at 22° C and terminated by the addition of KOH to 0.63 N. The activities of ribosomes in the presence of inhibitors were always compared to the activities of untreated controls examined concomitantly. The values recorded are averages of 2 to 4 experiments.

Streptogramin Group: PA114A and Vernamycin A

PA114A and vernamycin A, which are closely related in structure, bind directly to the peptidyl transferase locus of prokaryotic ribosomes (Vazquez, 1966a; Pestka, 1971; Ennis, 1965 and 1971). The peptidyl transferase locus of bovine 55S mitochondrial ribosomes is susceptible to the inhibitory action of PA114A and vernamycin A (Figure 6). The 39S large subribosomal particles are slightly more sensitive to these drugs than are the monosomes. The presence of 28S subunits in 55S monosomes may obstruct the binding sites for this class of antibiotics.

The 55S inhibition profile resembles the 70S profile more closely than that of 80S ribosomes. Low concentrations of PA114A and vernamycin A do not inhibit the peptidyl transferase activity of mitochondrial ribosomes to the same extent as that of crude 70S ribosomes. The sensitivity difference observed at low concentrations vanishes when the inhibition pattern of 55S ribosomes is compared to that of washed 70S ribosomes (Figure 7). When this is done, the inhibition patterns differ at high antibiotic concentrations. Since 55S mitochondrial ribosomes are not washed with 1 M NH_4Cl during their preparation, it is more appropriate to compare them to the crude bacterial ribosomes as in Figure 6.

Much scatter was observed in the antibiotic susceptibility profile of 70S ribosomes treated with PA114A or vernamycin A when different preparations of

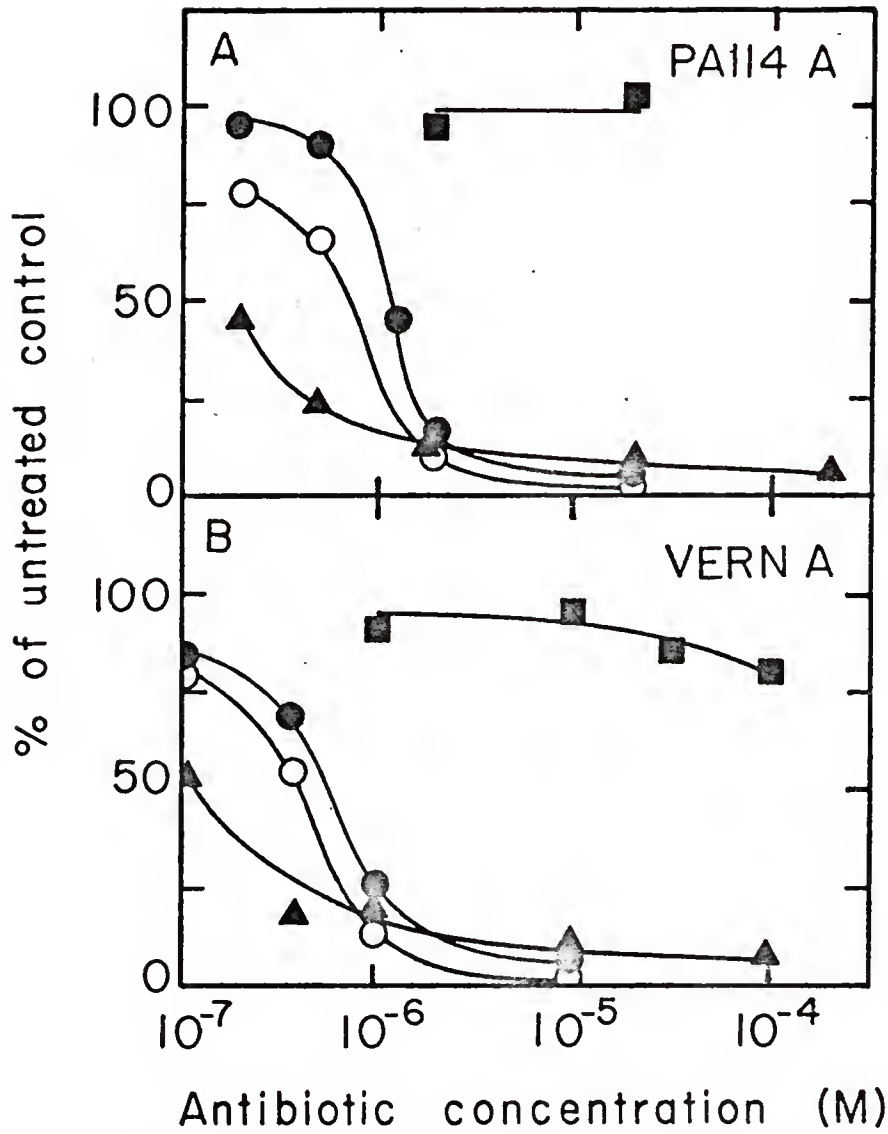


Figure 6. Effect of the streptogramin group of antibiotics, (A) PA114A, and (B) vernamycin A, on the peptidyl transferase activity of various ribosomes: ●——● mitochondrial 55S; o——o mitochondrial 39S; ▲——▲ crude E. coli 70S; and ■——■ microsomal 80S. Conditions are as in Figure 5.

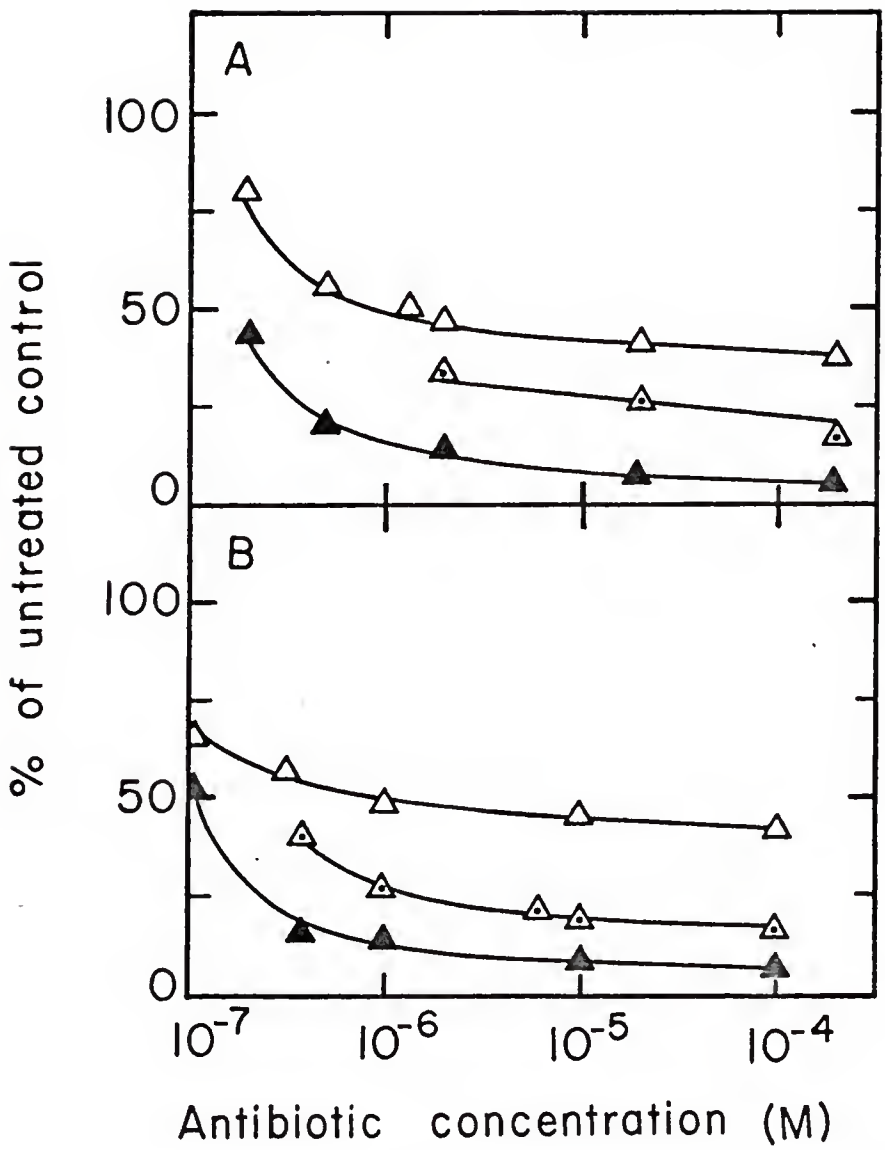


Figure 7. The susceptibility of crude and salt washed E. coli 70S ribosomes to inhibition by antibiotics of the streptogramin group, (A) PA114A, and (B) vernamycin A. Crude 70S E. coli ribosomes, ▲——▲ were prepared as described in "methods." These ribosomes were washed once, ▲——▲, or four times, Δ——Δ, with a high salt buffer (1 M NH_4Cl, 10 mM MgCl , 5 mM 2-mercaptoethanol, and 10 mM Tris-HCl, pH 7.5). Conditions are as in Figure 5.

ribosomes were used. It was postulated that the variability observed could be correlated with the effectiveness of the 1 M NH_4Cl wash on each ribosome preparation. To test this, crude and washed E. coli ribosomes were examined. The susceptibility of the crude 70S ribosomes to the streptogramins was gradually lost as the ribosomal particles were washed successive times with 1 M NH_4Cl (Figure 7). It seems likely that the strong binding of PA114A and vernamycin A requires proteins that are dislodged relatively easily from the ribosome. However, the antibiotic susceptibility profiles of crude and salt-washed 70S ribosomes treated with other antibiotics, for example lincomycin, are identical as discussed below. Apparently binding sites for the other antibiotics are held more tightly by 70S ribosomes.

It should be stressed that the inhibitory patterns for mitochondrial ribosomes treated with either PA114A or vernamycin A were reproducible. The binding site for the streptogramins may be more integrated into mitochondrial ribosomes than is the corresponding site in bacterial ribosomes.

Lincosamine Group: Lincomycin and Celesticetin

The peptidyl transferase center of mitochondrial ribosomes is sensitive to the lincosamines. Their susceptibility is clearly delineated in the antibiotic inhibition profile obtained at various concentrations of antibiotics

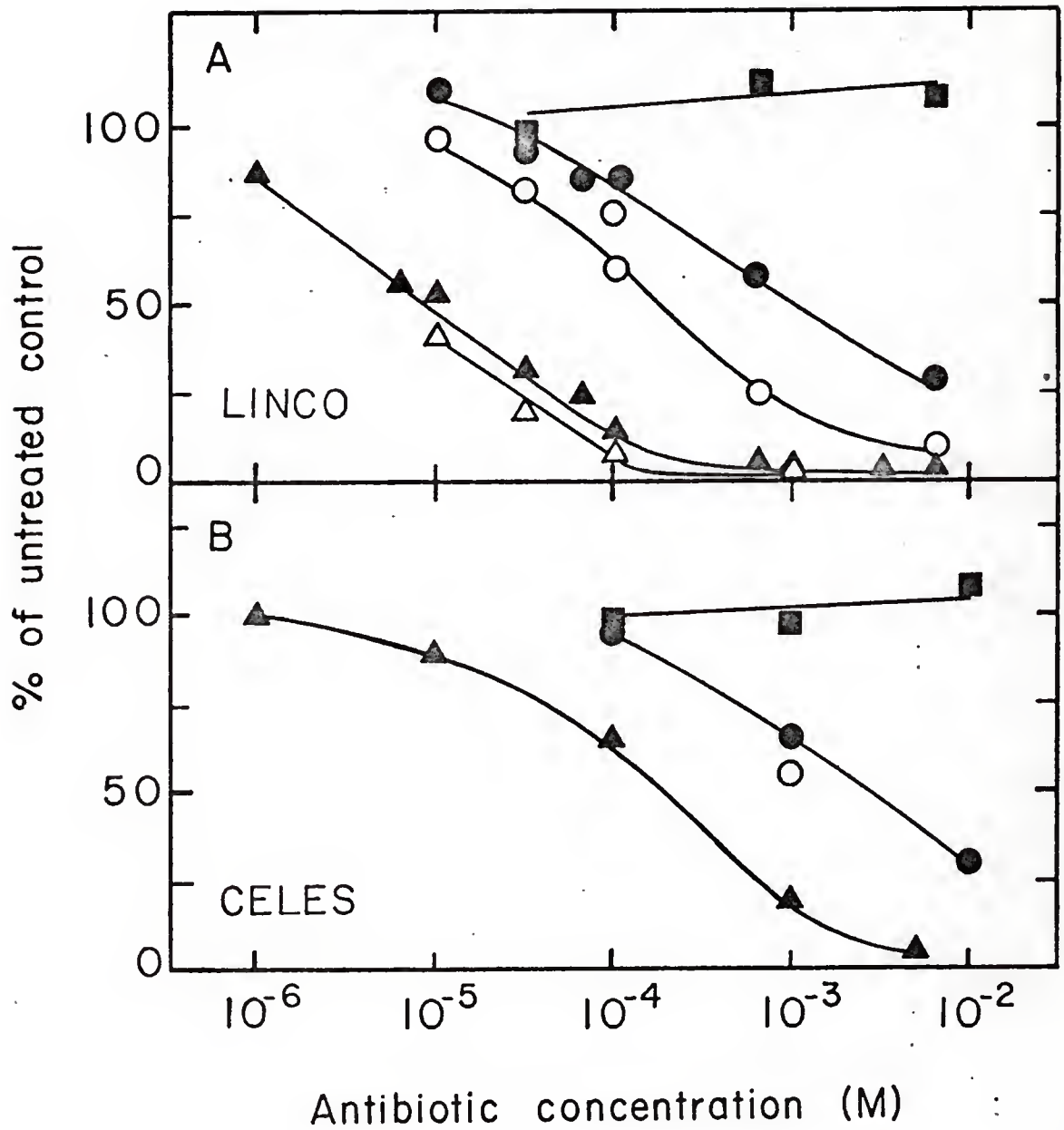


Figure 8. Effect of the lincosamine group of antibiotics, (A) lincomycin, and (B) celesticetin, on the peptidyl transferase activity of ribosomes: ●——●, mitochondrial 55S; o——o, mitochondrial 39S; ▲——▲, E. coli 70S; Δ——Δ, E. coli 50S; and ■——■, microsomal 80S. Conditions are as in Figure 5.

(Figure 8). By comparing to the E. coli 70S profile, one can notice that higher concentrations of antibiotics are required to inhibit the mitochondrial ribosomal system. In fact, 1 mM lincomycin and 2 mM celesticetin are required for 50% inhibition of the mitochondrial ribosomes in comparison to 0.01 mM lincomycin and 0.2 mM celesticetin needed for the same level of inhibition of E. coli ribosomes (Table 3).

When tested separately from the 55S monosome, the 39S large subribosomal particle of mammalian mitochondria appears to be more sensitive to lincomycin inhibition. The 39S particle requires 0.2 mM lincomycin to achieve 50% inhibition. This concentration is significantly higher than that required to inhibit bacterial ribosomes to the same extent. One can conclude that mitochondrial ribosomes are less sensitive than typical bacterial ribosomes to the action of the lincosamines.

The large 50S bacterial subunit is more susceptible to the effect of lincomycin than is the 70S monosome. This enhanced sensitivity is similar to the effect observed for 39S subribosomal particles as described above. It is possible that the presence of the small subunit in the monosome makes it more difficult for the lincomycin to bind to its site on the large subunit.

Macrolide Group: Carbomycin and Tylosin

It is well known that the macrolides exert an inhibitory action on protein synthesis by binding close to the site of peptidyl transferase on prokaryotic ribosomes (Pestka, 1971). Mammalian mitochondrial ribosomes have markedly reduced susceptibilities to both carbomycin and tylosin (Figure 9). Concentrations of 10^{-5} M carbomycin which inhibit the E. coli ribosomes by 90% have no effect on mitochondrial ribosomes. At this same concentration tylosin similarly does not affect mitochondrial ribosomes whereas it inhibits E. coli ribosomes by 50%. Higher concentrations of tylosin (300-fold) and carbomycin (700-fold) are required to inhibit the mitochondrial ribosomal reaction by 50% when compared to E. coli ribosomes (Table 3). The effect of the macrolides on the activity of 39S subunits parallels the observations made for 55S monosomes. Clearly, if mitochondrial ribosomes evolved from prokaryotic ribosomes, they have become less sensitive to these macrolides.

Controls for the Antibiotic Susceptibility Studies

Optimal Activity of Mitochondrial Ribosomes

Several investigators have proposed that ribosomes can exist in more than one state and that an equilibrium between the states exists in vivo (Chuang and Simpson, 1971; Schreier and Noll, 1971). The conformational

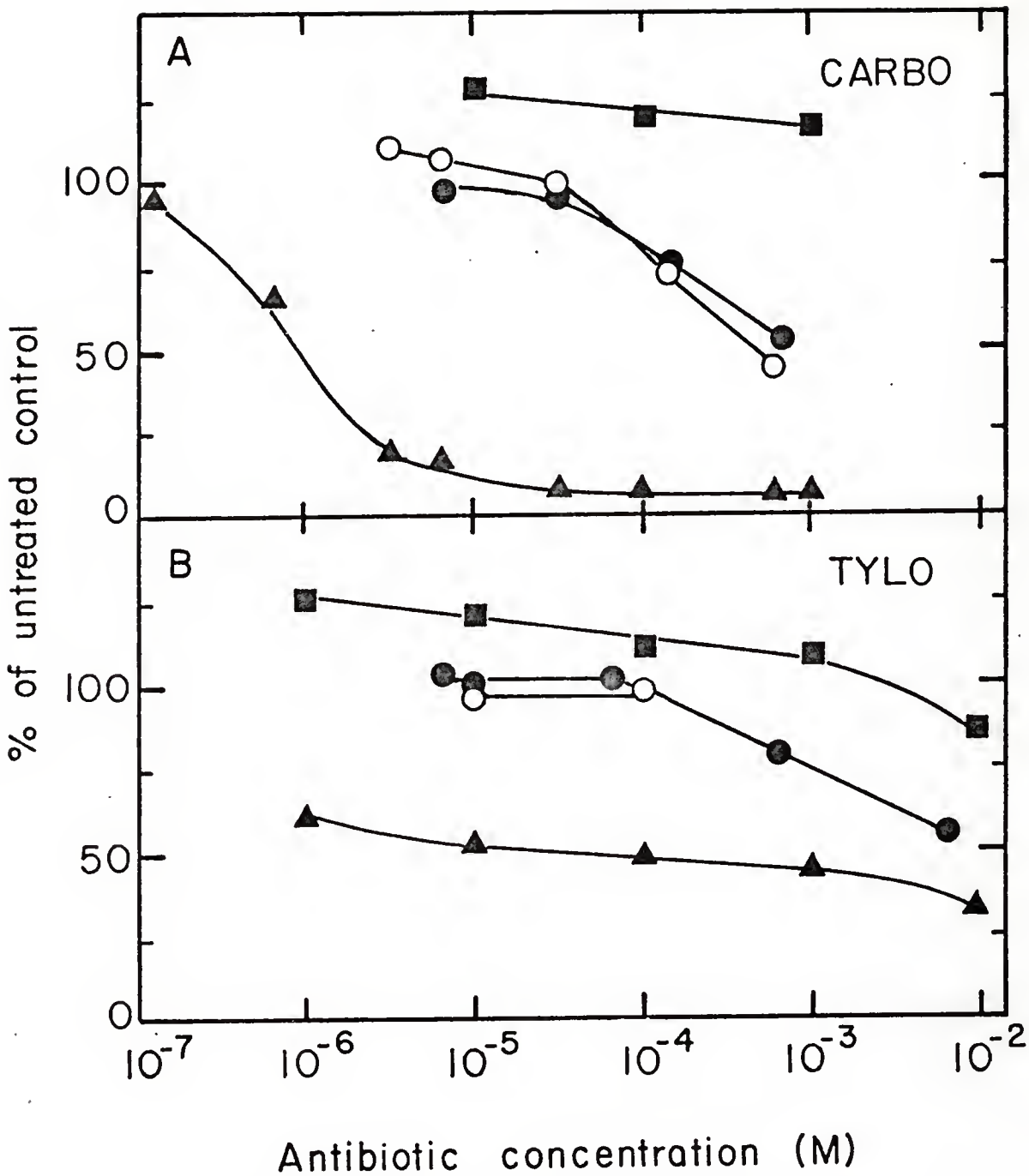


Figure 9. Effect of the macrolide group of antibiotics, (A) carbomycin, and (B) tylosin, on the peptidyl transferase activity of ribosomes: ●——●, mitochondrial 55S; o——o, mitochondrial 39S; ▲——▲, E. coli 70S; and ■——■, microsomal 80S. Conditions are as in Figure 5.

transitions of ribosomes going from one state to another may be the basis of their molecular function *in vivo* (Nishizuka and Lipmann, 1966; Spirin, 1969). These conclusions are supported by studies in which the ionic environment of ribosomes was varied (Miskin *et al.*, 1970; Zamir *et al.*, 1971; Zamir *et al.*, 1973; Ginzburg *et al.*, 1973). When ribosomes are isolated in a medium depleted of monovalent cations and low in Mg^{2+}, they become inactive in cell free assays. Activity can be fully restored by heating the ribosome to 40^{0} C in a medium containing the appropriate ionic conditions. Buffer conditions that maintain an intermediate level of activity have been used to establish this equilibrium *in vitro*. Under these conditions, 10^{-6} to 10^{-5} M concentrations of certain antibiotics, including those that affect peptidyl transferase activity, are capable of shifting the ribosomal equilibrium in favor of the active state (Miskin and Zamir, 1974).

In view of the diminished susceptibilities obtained to the lincosamines and the macrolides, it was important to demonstrate that the isolated mitochondrial ribosomes existed predominantly in an active form. If this were not the case, the addition of low concentrations of antibiotics could shift the equilibrium so that a higher percentage of active ribosomes would appear in the population and thus an apparent loss of susceptibility to the drugs would be observed. Mitochondrial ribosomes were isolated in a buffer containing 10 mM $MgCl_2$ and 100 mM KCl

(see "methods"). I did not expect to find a large percentage of inactive mitochondrial ribosomes in the preparations since Miskin and Zamir (1974) have shown that E. coli ribosomes are predominantly active when isolated in buffers containing at least 10 mM Mg^{2+} and 50 mM KCl.

In order to determine if there were any "inactive" mitochondrial ribosomes, incubation conditions shown by Miskin and Zamir (1974) to completely activate E. coli ribosomes (1 mM Mg^{2+}, 0.1 M NH_4Cl, 10 mM Tris, pH 7.5) were used (Table 5). As can be seen 70S control ribosome activity was only slightly increased by these conditions. Mitochondrial ribosomes on the other hand were not activated. The decrease in activity observed is probably due to nuclease activity operating at the high temperature (Appendix B and de Vries et al., 1971). The activity of the non-preincubated mitochondrial ribosomes compares well to that of E. coli ribosomes; therefore it is unlikely that a large percentage of these ribosomes are inactivated as they are isolated. Furthermore, the ionic conditions of the fragment reaction and the temperature (22° C) at which the reaction vessels are incubated are sufficient to activate ribosomes previously inactivated (Miskin and Zamir, 1974).

Absence of Bound Impurities to the Lincomycin Site of Mitochondrial Ribosomes

The diminished susceptibility of mitochondrial ribosomes to lincomycin could be due to the binding of a

TABLE 5

THE EFFECT OF PREINCUBATING RIBOSOMES AT 40° C
ON THE PEPTIDYL TRANSFERASE ACTIVITY

Ribosome		Preincubation	Activity (cpm)A_{260}	% of Un-incubated Control
E. coli	70S	none	522	100
	70S	40° C, 5 min	574	110
Mit	55S	none	546	100
	55S	40° C, 5 min	234	43

55S mitochondrial ribosomes and control 70S E. coli ribosomes were resuspended in a buffer containing 1 mM $MgCl_2$, 0.1 M NH_4Cl, 10 mM Tris-HCl, pH 7.5. Half of each sample was pre-incubated at 40° C for 5 min. The buffer was adjusted and fragment reactions were performed as described in "methods."

contaminant to or near the lincomycin site thereby partially occluding this site. Two control experiments were performed to examine this possibility.

(1) If a contaminant were bound to mitochondrial ribosomes it also would be present in the high speed supernatant obtained during the preparation of these ribosomes. It was postulated that if the contaminant were nonspecific it might bind to E. coli ribosomes as well. 70S E. coli ribosomes were resuspended in the mitochondrial high speed supernatant in order to scavenge any such contaminants. After 30 minutes the 70S ribosomes were concentrated by centrifugation and used directly in the fragment reaction. The susceptibility of E. coli ribosomes to lincomycin was not altered by this treatment (Table 6). This experiment does not rule out specific binding of the contaminant to mitochondrial ribosomes.

(2) Mitochondrial ribosomes were examined directly to determine whether a loosely bound contaminant was responsible for the diminished inhibitory action of lincomycin. 39S mitochondrial ribosomes were washed with buffer Z (20 mM triethanolamine, pH 7.5; 5 mM $MgCl_2$; 0.5 M KCl; 5 mM 2-mercaptoethanol) and then centrifuged through a 4 ml layer of buffer Z containing 20% sucrose in order to separate the washed ribosomes from any dislodged proteins. This treatment is capable of removing some ribosomal proteins from mitochondrial ribosomes (David Matthews, unpublished observation). The resulting

TABLE 6

LINCOMYCIN SUSCEPTIBILITY OF E. COLI RIBOSOMES RESUSPENDED IN MITOCHONDRIAL SUPERNATANT

Ribosomal Particles	Treatment	Lincomycin Concentration		
		10^{-3}M	10^{-4}M	10^{-5}M
		% of Control		
E. coli 70S	Mit. Supt.	2	19	57
70S	---	2	16	59

2 A_{260} units of E. coli ribosomes were resuspended into 6 ml of postribosomal mitochondrial supernatant. The mixture was stirred for 30 min. The 70S ribosomal particles were concentrated by centrifugation at 230,000 x g for 3 hrs in a Spinco type 65 rotor. They were resuspended directly into the fragment reaction incubation mixture. The control 70S ribosomes were the standard 1 M NH_4Cl washed ribosomes used in all the inhibition studies. The reaction conditions are described in "methods."

"core" particles were examined for their susceptibility to lincomycin (Table 7). A slight increase in susceptibility to this drug is observed for the core particles. This change is not significant when compared to the antibiotic profile of E. coli ribosomes. It appears that the diminished susceptibility of mitochondrial ribosomes to lincomycin is not due to a bound contaminant but that this reflects an intrinsic property of mitochondrial peptidyl transferase.

The Effect of Lincomycin on Salt-Washed E. coli Ribosomes

Crude (buffer S) and salt-washed (buffer Z) E. coli ribosomes are inhibited to the same extent by several concentrations of lincomycin (Table 7). An identical effect was obtained when E. coli ribosomes washed with 1 M NH_4Cl were used (Figure 8). It appears that the binding site for lincomycin is more tightly held by E. coli ribosomes than that for the streptogramins (Figure 7).

Reproducibility of Results

When this study was initiated low yields (5 to 10 A_{260} units) of mitochondrial ribosomes were obtained from each preparation. Antibiotic inhibition profiles were constructed by averaging 2 to 3 experimental points. The effect of antibiotics on ribosomes prepared on different days was reproducible. The maximum error did not exceed 10% of the untreated control for any antibiotic

COMPARISON OF LINCOMYCIN SUSCEPTIBILITY
OF CRUDE AND SALT-WASHED RIBOSOMES

Ribosomal Particles	Wash Condition	Lincomycin Concentration				
		10^{-3}	10^{-4}	3.3×10^{-5}	10^{-5}	10^{-6}
		% of Untreated Control				
Mitochondrial 55S	DVT	50	85	92	110	-
39S	DVT	20	70	90	97	-
39S	Z	-	60	-	-	-
E. coli 70S	S	0	10	-	50	81
70S	Z	1	8	-	46	86
50S	S	0	7	-	45	-
50S	Z	0	6	-	32	-

Mitochondrial 55S and 39S ribosomes were prepared through sucrose density gradients in DVT buffer (10 mM $MgCl_2$, 0.1 M KCl; 10 mM Tris-HCl, pH 7.6, 5 mM 2-mercaptoethanol, 0.1 mM EDTA). 0.8 A_{260} units of each were examined for sensitivity to lincomycin. 5 A_{260} units of 55S ribosomes were washed and dissociated in Z buffer (5 mM $MgCl_2$, 0.5 M KCl, 20 mM triethanolamine, pH 7.5, and 5 mM 2-mercaptoethanol) and centrifuged through sucrose density gradients in that condition. The material sedimenting at 39S was collected and examined. 1.2 A_{260} units per reaction mixture was used. Crude *E. coli* ribosomes were centrifuged through sucrose density gradients in S buffer (10 mM $MgCl_2$, 0.1 M KCl, 20 mM triethanolamine, pH 7.6 and 5 mM 2-mercaptoethanol) or Z buffer. Material sedimenting at 50S and 70S in each gradient was collected. 0.6 to 1.2 A_{260} units of ribosomes were used. The conditions for the fragment reaction are described in "methods."

concentration tested and often varied only by 2 to 3%. Significant differences between responses to the antibiotics by the various ribosomes are those which are greater than 10% of the controls.

The presence of cytoplasmic 60S ribosomes in preparations of 55S mitochondrial ribosomes isolated by procedure 1 did not affect the measured activity by more than 2 to 3%. This contamination did not significantly alter the inhibition patterns observed. Indeed, all the antibiotic inhibition patterns for mitochondrial ribosomes were confirmed with ribosomes prepared by procedure 2 (see "methods").

The Function of Mitochondrial Ribosomes in Additional Assays

The functional activity of mitochondrial ribosomes was examined by two additional assays in order to demonstrate the integrity of the particles used for the peptidyl transferase assay. These assays included the poly U-dependent incorporation of [^{3}H]phenylalanine and the [^{3}H]GTP binding ability of mitochondrial ribosomes. The poly U-dependent assay was a convenient and standard method for showing an intact and functional ribosome and the GTP binding assay was used to demonstrate that mitochondrial ribosomes bind GTP in near stoichiometric amounts in a manner analogous to *E. coli* ribosomes.

Poly U-Dependent Incorporation of [^{3}H] Phenylalanine

Mitochondrial ribosomes isolated from bovine liver are able to polymerize phenylalanine when assayed in standard poly-U directed cell free systems (O'Brien et al., 1974). As shown in Table 8, the mitochondrial ribosomes depend on added soluble factors and poly U for maximal activity. The lower activities obtained for mitochondrial ribosomes when compared to bacterial ribosomes; 850 pmoles phenylalanine/mg RNA in 15 min for rat mitochondrial ribosomes (Table 8) compared to 8085 pmoles phenylalanine/mg RNA in 15 min for E. coli ribosomes may be due to the nature of the mitochondrial system. Nascent proteins attached to the isolated 55S mitochondrial ribosomes (O'Brien, 1971) are probably of a "very sticky," hydrophobic nature and may not be easily dislodged from the ribosomes. This property of the nascent proteins may not allow for proper ribosome run off and reinitiation of protein synthesis even though the ribosomes are incubated for 5 min in complete medium before the addition of poly U.

Mitochondrial factors are adequate to support mitochondrial protein synthesis (experiments 1 and 3a), but most homologous factor preparations work less well than those prepared from E. coli (experiments 2a and 3a). Nevertheless, activities were obtained with 0.1 to 0.2 mg protein/ml mitochondrial factors. Although 0.1 mg protein/ml E. coli factors were sufficient to saturate control assays using E. coli ribosomes (see Appendix B), maximal activities with

TABLE 8

ACTIVITY OF BOVINE MITOCHONDRIAL RIBOSOMES IN POLY U-DEPENDENT SYSTEMS

Exp.	Ribosome		Factors	Concentration (mg/ml)	[^{3}H]Phe incorporated pmoles/mgRNA in 15 min
1	Cow 55S	complete	Mitochondria	0.1	329
			none	-	16
2(a)	Cow 55S	complete	E. coli	1.6	561
		-Poly U	"	1.6	231
		+Puromycin (100 µg/ml)	"	1.6	151
			none	-	0
(b)	Cow 28S+39S	complete	E. coli	1.6	1120
		-Poly U	"	1.6	879
		+Puromycin (100 µg/ml)	"	1.6	152
			none	-	173
3(a)	Rat 55S	complete	Mitochondria	0.2	297
		complete	E. coli	0.85	850
			none	-	0
(b)	Rat 28S+39S	complete	Mitochondria	0.7	1624

The results are corrected for the minus ribosome controls at 15 min determined for each experiment as follows: exp 1, 50 pmoles/mgRNA; exp 2, 130 pmoles/mgRNA; and exp 3, 150 pmoles/mgRNA.

Bovine and rat liver mitochondrial ribosomes were prepared through sucrose density gradients in T buffer as described in "methods." The quantities of ribosomes used in each experiment were as follows: exp 1, 0.5 A_{260} units 55S; exp 2, 0.5 A_{260} units 55S, 0.2 A_{260} units 28S, 0.2 A_{260} units 39S; exp 3, 0.5 A_{260} units 55S, 0.3 A_{260} units 28S and 0.3 A_{260} units 39S. The reaction conditions are described in "methods."

mitochondrial ribosomes were only obtained when 0.8 mg protein/ml E. coli factors were used. The level of activity obtained with the mitochondrial factors was always lower than that obtained with bacterial factors. For example, in experiment 3a the homologous mitochondrial system incorporated 297 pmoles phenylalanine/mg RNA in 15 min. The value for the heterologous system was 850 pmoles (Table 8). The low activity of mitochondrial ribosomes in the presence of homologous factors appears to be due to the presence of an inhibitor in these factor preparations, as described in Appendix B.

The polymerization of phenylalanine by 55S mitochondrial ribosomes is dependent on the presence of poly U in the reaction mixture and is inhibited by the addition of 100 µg/ml puromycin. As seen in experiment 2 (Table 8), the omission of poly U results in lowering the incorporation of phenylalanine by 60%, and puromycin inhibits this reaction by 73%.

Mitochondrial native subribosomal particles also are active in the poly U dependent system (experiments 2b and 3b, Table 8). However, they have high activities (879 pmoles/mgRNA in 15 min) in the absence of poly U. This endogenous activity is not due to a non-protein synthesizing side reaction since it can be inhibited by 100 µg/ml puromycin to 152 pmoles phenylalanine/mgRNA in 15 min. This observation can possibly be explained by having a natural message either bound to one of the

mitochondrial subunits or simply cosedimenting with either subunit in the gradient. If so, it is probably bound to the small subunit in initiation complexes as is observed for other ribosomal systems. Alternatively, some of the native subunits normally found may have been derived from dissociation of ribosome couples early in the process of protein synthesis, retaining their mRNA.

Generally, mitochondrial ribosomes obtained from rat liver were more active in the poly U dependent system than those obtained from bovine liver. This may be due to the longer preparation time required for the bovine 55S ribosomes and because the animals used were much older than the rats.

In summary, 55S mitochondrial ribosomes are active in a poly U dependent system and absolutely require the addition of either homologous mitochondrial factors or heterologous bacterial factors. The recombined subunits are active as well.

Function of Mitochondrial Ribosomes in the Binding of [^{3}H]GTP

As another measure of mitochondrial ribosomal function, we examined their capacity to bind [^{3}H]GTP. From studies in other ribosomal systems (Nishizuka and Lipman, 1966; Bodley *et al.*, 1970), it is known that isolated ribosomes can bind [^{3}H]GTP only if they contain bound EF-G, the translocase elongation factor. It is, therefore, of interest to study this partial reaction of

protein synthesis to elucidate the possible involvement of a translocase type of elongation factor in the functioning of mammalian mitochondrial ribosomes.

As seen in Table 9, both mitochondrial monosomes (55S), and combined 28S and 39S subribosomal particles, as normally prepared through sucrose density gradients in DVT-30 buffer containing 30 mM Mg^{2+} and 0.07 M KCl, bind [^{3}H]GTP well. This result suggests that the isolated mitochondrial ribosomes contain bound EF-G.

Under the conditions of this assay, the 55S ribosomes bind about 3.6 pmoles of [^{3}H]GTP/5 pmoles* of ribosomes. This value is better than that obtained with E. coli ribosomes, 2.4 pmoles/5 pmoles ribosomes. Fusidic acid (3 mM) is required to stabilize the E. coli ribosome-EF-G-GTP complex. The conditions of the millipore filtration technique are such that only half the complexes formed in free solution are reproducibly measured (Bodley et al., 1970). Thus, a maximum of 2.5 pmoles [^{3}H]GTP bound/5 pmoles active E. coli ribosomes would be detected by this method. The mitochondrial ribosome-factor-GTP complex, on the other hand, is not unstable in the absence of fusidic acid. [^{3}H]GTP binding reactions performed in the absence of fusidic acid gave the same results for mitochondrial ribosomes. Therefore, the millipore filtration technique probably detects all the complexes

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* See Appendix A for determination of pmoles.

TABLE 9

BINDING OF [^{3}H]GTP TO RIBOSOMES

Ribosomal Particles	Wash Buffer	mM Mg^{2+} / M Salt	pmoles [^{3}H]GTP bound per 5 pmoles ribosomes[a]
Mitochondrial			
Monoribosome (55S)	DVT-30	30/0.07[b]	3.58
Subribosomal particles (native)	DVT-30	30/0.07[b]	4.78
Subribosomal particles (derived)	NCB	20/1[c]	1.08
"	NCB	20/1[c] (reconstituted)	3.60
"	NCC	20/2[c]	0.06
E. coli			
Monoribosome (70S)	SGB	10/0.05[c]	2.40
"	W	10/1[c]	0

[a]All values corrected for the minus ribosome control (0.07 pmoles [^{3}H]GTP).
[b]Salt = KCl.
[c]Salt = NH_4Cl.

Bovine liver mitochondrial monosomes and native subribosomal particles were prepared through sucrose density gradients in DVT-30 buffer (10 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 0.07 M KCl, 6 mM 2-mercaptoethanol). Derived subribosomal particles were obtained by treating 55S monosomes with the following buffers: NCB, 10 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 1 M NH_4Cl, 5 mM 2-mercaptoethanol; and NCC, 10 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 2 M NH_4Cl, 5 mM 2-mercaptoethanol. The derived particles were centrifuged through sucrose density gradients in these buffers. 6.7 pmoles 55S, 3 pmoles native subribosomal particles, and 4.7 pmoles derived subribosomal particles were used. 6 pmoles E. coli ribosomes in SGB buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.05 M NH_4Cl, and 5 mM 2-mercaptoethanol) or washed in W buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 M NH_4Cl, and 5 mM 2-mercaptoethanol were used as controls. All ribosomes were resuspended directly into the reaction mixture. Reaction conditions are described in "methods."

formed in free solution. On this basis, isolated mitochondrial ribosomes can bind [^{3}H]GTP in near stoichiometric amounts.

The ability of E. coli ribosomes to bind [^{3}H]GTP can be diminished by repeated washes in buffers containing 1 M NH_4Cl (Nishizuka and Lipmann, 1966). This procedure serves to deplete E. coli ribosomes of their bound EF-G. To determine whether the [^{3}H]GTP binding activity of mitochondrial ribosomes was due to the presence of an analogous factor which could be stripped from the ribosomes by washes in high salt buffers, mitochondrial ribosomes were prepared through sucrose density gradients in these buffers. When mitochondrial subribosomal particles are prepared in the presence of NCB buffer, containing 1 M NH_4Cl, their capacity to bind [^{3}H]GTP is reduced (77%) to 1.08 pmoles/5 pmoles of ribosomes (Table 9). This activity can be abolished by washing the subribosomal particles in NCC buffer, containing 2 M NH_4Cl. These results suggest that mitochondrial ribosomes, like other ribosomes, contain a bound EF-G type of elongation factor. This factor furthermore begins to be stripped from mitochondrial ribosomes under conditions which are effective in removing EF-G from E. coli ribosomes (Table 9 and Nishizuka and Lipmann, 1966; Bodley et al., 1970). Significantly, the capacity to bind [^{3}H]GTP can be reconstituted by adding the removed factors to the depleted subribosomal particles followed by dialysis in 10 volumes of DVT-30 buffer to remove the high salt (Table 9).

CHAPTER 4
DISCUSSION

Significance of Results

Peptidyl Transferase Activity of Mitochondrial Ribosomes

Because of the complexity of ribosomes, a detailed understanding of the structure-function relationships within these macromolecules is slow in coming into existence. Several approaches have been used to elucidate many of the functions of the individual ribosomal proteins: (a) mutation of ribosomal proteins, (b) reconstitution of particles missing one protein, and (c) interaction of inhibitors with specific proteins.

The fragment reaction is a convenient and direct way to study, specifically, the peptidyl transferase activity of mitochondrial ribosomes with a limited interference from other ribosomal activities. Mitochondrial ribosomes contain a functional peptidyl transferase center as is demonstrated by their activity in the fragment reaction. As with all other ribosomes, the peptidyl transferase activity is an integral function of the large subunit. Therefore, at a coarse level of analysis, the peptidyl transferase locus of mitochondrial ribosomes is similar to that found on other ribosomes. In the modified fragment reaction, an entire molecule

of amino acyl-tRNA rather than a fragment produced by RNase T_1 digestion is used. In this case, a small subunit may be required to lend greater stability to the binding of the entire amino acyl-tRNA. This requirement may account for the stimulation of peptidyl transferase activity observed in the presence of both subunits.

Antibiotic Susceptibility

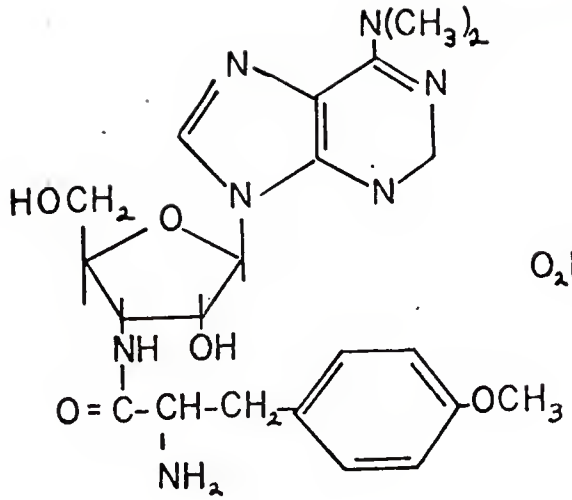
The fragment reaction can be divided into three steps, as pointed out by Pestka (1969): (a) binding of the peptidyl-tRNA to the "P" site of the peptidyl transferase center; (b) binding of puromycin, an analogue of amino acyl-tRNA, to the "A" site, and (c) the formation of a peptide bond between the amino acid moiety of the peptidyl-tRNA and puromycin. Antibiotic probes can affect any one of the steps either by binding directly to proteins within each site or by causing conformational changes when bound to a distant site. The binding of antibiotic inhibitors to ribosomal proteins is known to be highly specific (reviewed in: Pestka, 1971; Pongs et al., 1974). Eventually a detailed map of protein synthesis will be made possible by probing the substrate binding sites with antibiotics.

Several early studies demonstrated the susceptibility of mitochondrial protein synthesis to prokaryotic specific inhibitors but not to inhibitors acting specifically on eukaryotic ribosomes (Lamb et al., 1968;

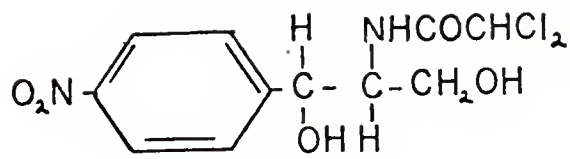
Freeman, 1970; Kroon and de Vries, 1971; de Vries et al., 1971). For this reason, several antibiotics known to have a strong inhibitory effect on bacterial protein synthesis were chosen to probe the peptidyl transferase site of mitochondrial ribosomes.

Structures of representative antibiotics used in this study appear in Figure 10. They have molecular weights of 300 to 1100 gm/mole. Their size permits them to bind specifically to ribosomal proteins without greatly perturbing ribosomal structure. Binding sites on E. coli ribosomes have been described for each antibiotic (reviewed in: Vazquez, 1974; Pestka, 1971). In particular, the binding site for chloramphenicol has been thoroughly investigated (Nierhaus and Nierhaus, 1973; Pongs et al., 1973; Dietrich et al., 1974). The ribosomal binding sites for other antibiotics have been related to the chloramphenicol binding site by competition experiments (Vazquez, 1966a; Pestka, 1974; Vazquez et al., 1973; Vazquez and Monro, 1967). These experiments have been the basis for speculations about the spatial interrelationship of the peptidyl transferase locus of bacterial ribosomes. It was hoped that a direct comparison of the antibiotic susceptibilities of mitochondrial and bacterial ribosomes would lead to the elucidation of the mitochondrial ribosomal locus for peptidyl transferase.

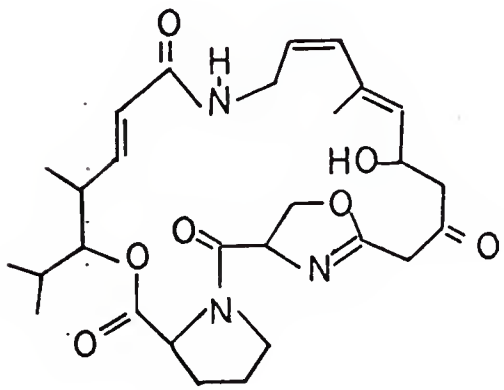
Figure 10. Chemical structures of inhibitors. The structures for puromycin, chloramphenicol, streptogramin A and lincomycin were obtained from Bücher and Sies, 1969. The schematic structures for carbomycin and tylosin were obtained from Gale et al., 1972.



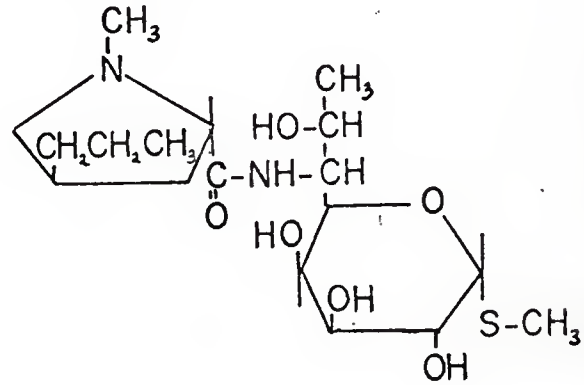
Puromycin



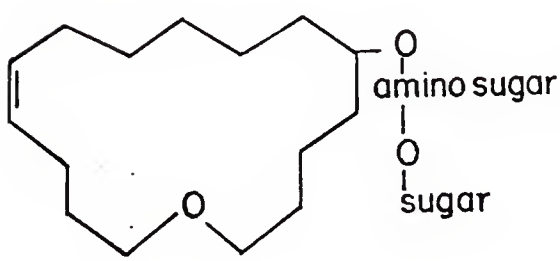
D(-) Threo Chloramphenicol



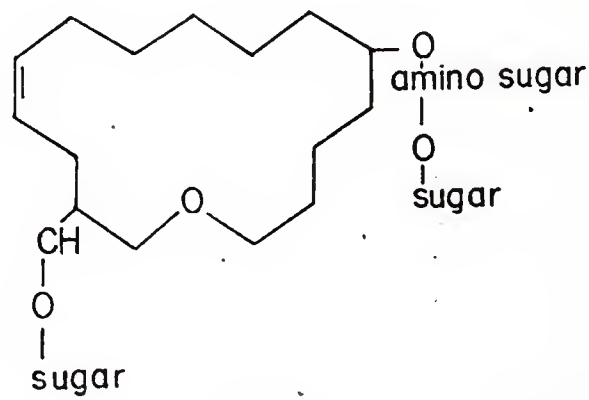
Streptogramin A



Lincomycin



Carbomycin



Tylosin

Our results with isolated E. coli ribosomes reproduced results reported in the literature (Monro and Vazquez, 1967; Contreras et al., 1974). Mammalian mitochondrial ribosomes were inhibited to the same extent as E. coli ribosomes by moderate, that is 1 to 100 μM, concentrations of chloramphenicol and the streptogramin group of antibiotics. Significant differences in their responses to other antibiotics were observed. Antibiotics of the lincosamine and macrolide groups were required at concentrations one to two orders of magnitude higher to inhibit the reaction by 50% than was necessary for similar inhibition of the 70S ribosomes (Table 3). The inhibition patterns obtained with each of the antibiotic groups will be discussed and their significance for the understanding of protein synthesis within mammalian mitochondria delineated in turn.

Chloramphenicol

Chloramphenicol (CAP) acts to prevent the binding of substrates to the "A" site of the peptidyl transferase locus of prokaryotic ribosomes (Pestka, 1969; Pestka, 1970). Monoiodoamphenicol, a synthetic analogue of CAP has been used as an affinity label to identify protein L_{16} of the large E. coli subunit as the CAP binding site (Pongs et al., 1973). Another approach, that of partial reconstitution of the ribo-

somes, has also been used to identify this binding site (Nierhaus and Nierhaus, 1973). The specificity of CAP binding to ribosomes is emphasized by the fact that only the D-threo configuration will bind (Vazquez, 1966a).

In this study, it is shown that CAP acts directly on the mitochondrial ribosomes (Figure 5). Concentrations of the same order of magnitude required to inhibit bacterial ribosomes by 50% inhibited mitochondrial ribosomes to the same extent (Table 3). In this respect both types of ribosomes seem very similar. As expected, CAP does not inhibit cytoplasmic 80S ribosomes (Figure 5 and de Vries et al., 1971). This indicates a high degree of homology between mammalian mitochondrial ribosomes and bacterial ribosomes.

The CAP binding site of bacterial ribosomes appears to be highly conserved since no chloramphenicol resistant mutants with a lesion at the ribosomal level have been isolated (Benveniste and Davies, 1973; O'Brien and Matthews, 1975). However, mitochondrial ribosomes resistant to chloramphenicol have been isolated from yeast (Grivell et al., 1973) and HeLa cells (Spolsky and Eisenstadt, 1972). These mutants will be important for studies elucidating the nuclear-mitochondrial interactions occuring in mitochondrial biogenesis.

The susceptibility of mitochondrial protein synthesis to CAP has been known since 1959 (Rendi, 1959). Mitochondria of all eukaryotes are similarly affected by this drug. In past years, CAP has been the tool used to discriminate protein products of mitochondrial origin from those of cytoplasmic origin (Clark-Walker and Linnane, 1966). In particular several subunits of the cytochrome oxidase (Mason and Schatz, 1973; Clark-Walker and Linnane, 1966; Schatz and Mason, 1974; de Vries and Kroon, 1970) and several subunits of the oligomycin-sensitive ATPase (Tzagoloff and Meagher, 1972) have been identified as mitochondrial products. Studies with intact mitochondria show no membrane barrier to the penetration of the drug.

The possibility that CAP had a direct effect on oxidative phosphorylation in addition to its effect on protein synthesis was raised by several investigators. In these studies, the administration of CAP to intact mitochondria resulted in a dysfunction of some of the cytochrome systems. The site of action of CAP within mitochondria was not resolved until recently (de Vries et al., 1971). Nijhof and Kroon (1974) argued that the effect observed on energy production was indirectly caused by a primary effect on mitochondrial protein synthesis. Since mitochondrial products are necessary for the operation and integration of the electron

transport system within the mitochondria, this argument can explain the toxic action of chloramphenicol on tissues that are rapidly regenerating. Daughter cells in these tissues would contain functionally deficient mitochondria (Kroon et al., 1973; O'Brien and Matthews, 1975). For example, elevated concentrations of CAP cause reversible bone-marrow depression in man.

PA114A and Vernamycin A

PA114A and vernamycin A, which are closely related in structure, are potent inhibitors of protein synthesis on prokaryotic ribosomes (Vazquez, 1974). They have been shown to interact directly with the 50S subunit of prokaryotic ribosomes by inhibiting the binding of the amino acyl end of amino acyl-tRNA to the "A" site (Ennis, 1966; Mao and Putterman, 1968; Vazquez, 1966b). Only one binding site per 50S subunit of the ribosome has been detected for vernamycin A (Ennis, 1971). Optimal binding of vernamycin A (Ennis, 1971) occurs at concentration of K^+ and Mg^{2+} found in the fragment reaction.

Mitochondrial ribosomes are quite susceptible to the inhibitory action of antibiotics from the streptogramin A group (Figure 6). The concentration required to inhibit the peptidyl transferase reaction by 50% is of the same order of magnitude for both bacterial and mitochondrial ribosomes.

The inhibition profile of mitochondrial ribosomes treated with these antibiotics is sigmoidal in shape. This may indicate a conformational change in the ribosome caused by binding of these antibiotics. Miskin et al. (1974) have observed conformational changes in E. coli ribosomes when these were treated with antibiotics specific for peptidyl transferase. Conformational changes also have been observed when vernamycin B interacts with these ribosomes (Ennis, 1971).

Competition experiments were utilized to relate the binding site of the streptogramins to that of CAP. Although vernamycin A can inhibit CAP binding to ribosomes (Vazquez, 1966a), the reverse was not observed (Ennis, 1971). The ribosomal protein to which the streptogramins bind has not yet been identified. This protein probably is not tightly bound to the ribosome since it is easily removed by 1 M NH_4Cl (Figure 7). The binding sites for CAP and the streptogramins are probably distinct but overlapping in bacterial ribosomes. Since the inhibition profiles obtained with both CAP and the streptogramins for mitochondrial ribosomes are similar to those obtained for bacterial ribosomes, it is likely that the CAP and streptogramin binding sites in mitochondrial ribosomes overlap as well.

Lincomycin and Celesticetin

Antibiotics of the lincosamine group inhibit substrate binding to the "A" and "P" sites of the peptidyl transferase center of prokaryotic type ribosomes (Chang et al., 1966; Pestka, 1971). Studies by Vazquez et al. (1973) have indicated only one binding site for lincomycin on bacterial ribosomes. On the basis of competition experiments, the lincomycin binding site is closely related to, but distinct from, the binding sites for CAP and the macrolides.

Although this group of antibiotics can inhibit the peptidyl transferase activity of mammalian mitochondrial ribosomes, very high concentrations are required to inhibit the reaction by 50% as compared to the bacterial system (Table 3). Lincomycin resistance is also observed in poly U directed systems (Ibrahim et al., 1974). The abnormally high concentrations of the drug required to inhibit the reaction indicate that the binding site of mitochondrial ribosomes is not similar to that of bacterial ribosomes. Concentrations which inhibit mitochondrial peptidyl transferase do not inhibit the activity of 80S microsomal ribosomes. Therefore, the inhibitory effect is specific and may reflect a loss of affinity of the ribosomal protein for lincomycin.

The possibiltiy of phylogenetic differences among mitochondrial ribosomes was raised by Linnane

and coworkers (Firkin and Linnane, 1969; Towers et al., 1972), who showed marked differences in the concentrations of several antibiotics, including lincomycin, required to inhibit protein synthesis by isolated intact mitochondria. Kroon and de Vries (1971), however, attributed these observed differences to the impermeability of mammalian mitochondrial membranes. When the mitochondria were osmotically shocked, protein synthesis was inhibited by these drugs. Linnane, repeating his experiments with osmotically shocked mitochondria, maintained his argument (Towers et al., 1973). The similarity of their data is not obvious until one examines the results of both groups relative to the lincomycin inhibition profile obtained in this study (Figure 11; Denslow and O'Brien, 1974). Inhibition data obtained by Grivell et al. (1971b) for intact yeast mitochondria are plotted for comparison. One can conclude that despite suggestions of a membrane permeability barrier to lincomycin, the isolated mitochondrial ribosomes are partially resistant to the drug. The comparison illustrated in Figure 11 stresses the importance of using isolated ribosomes in studies of antibiotic susceptibilities. These results are significant in understanding the effect of lincomycin on mammalian cells in vivo. It has been suggested that the impermeability of mitochondrial membranes to

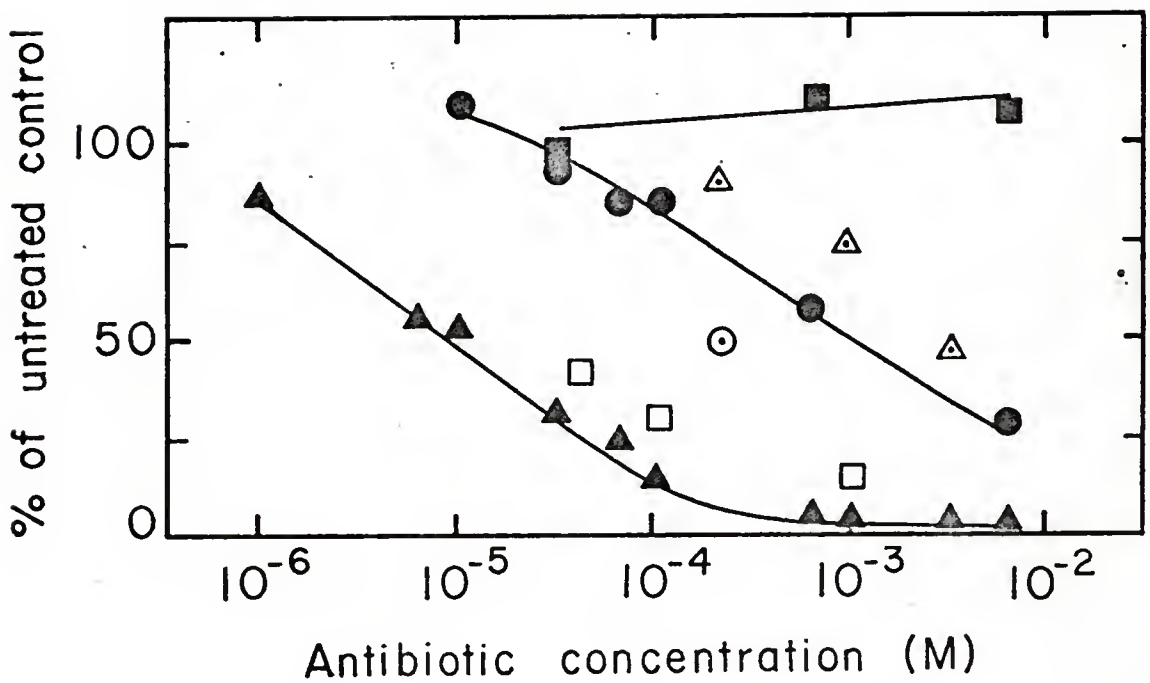


Figure 11. Effect of lincomycin on peptidyl transferase activity of ribosomes: a comparison of my results with relevant data from other laboratories. ●——●, 55S mitochondrial; ▲——▲, 70S E. coli, and ■——■, 80S cytoplasmic ribosomes were examined as described in Figure 5. □, inhibition of protein synthesis in intact yeast mitochondria (Grivell et al., 1971). △, inhibition of protein synthesis in osmotically shocked rat liver mitochondria according to Kroon and de Vries (1970). ⊙, inhibition of protein synthesis in osmotically shocked rat liver mitochondria according to Towers et al. (1973).

lincomycin (Kroon and de Vries, 1971; Kroon et al., 1973) is responsible for their resistance to this antibiotic. Results in this study indicate that this may not be the case since very high levels of lincomycin are needed to inhibit the isolated mitochondrial ribosomes.

Carbomycin and Tylosin

The macrolide antibiotics contain large lactone rings of 12 to 22 atoms and are thus structurally related (Pestka, 1971). It is thought that all macrolides bind to one major site on the ribosome but that the interactions with this site vary according to the size of the lactone ring (Pestka, 1971; Mao and Robishaw, 1971). For example, the larger macrolides such as carbomycin and tylosin inhibit peptide bond formation, whereas the smaller macrolides such as erythromycin do not. Even though erythromycin cannot inhihit peptidyl transferase activity, it can still interfere with the binding of CAP to ribosomes, thereby indicating that it binds to a site close to the CAP binding site.

In this study we have demonstrated that the peptidyl transferase activity of mammalian mitochondrial ribosomes shows a greatly diminished susceptibility to carbomycin and tylosin (Figure 9). High antibiotic concentrations, 0.7 mM carbomycin and 10 mM tylosin, which begin to inhibit the microsomal 80S ribosomes, are required to inhibit bovine liver mitochondrial

ribosomes by 50%. This may represent a nonspecific inhibition. Similar effects were noted in isolated rat liver mitochondrial ribosomes as well (de Vries et al., 1973; Kroon et al., 1974). In experiments recently published by Ibrahim et al. (1974), isolated rat liver mitochondrial ribosomes examined in a poly U dependent system required a 0.1 mM concentration of carbomycin to inhibit the reaction by 50%. The authors chose to interpret their results as indicating a high susceptibility of the isolated mitochondrial ribosomes to carbomycin. However, if their data are compared to the antibiotic profile in Figure 9, it is clear that their mitochondrial ribosomes are not much more susceptible to carbomycin than those reported here.

These results differ sharply from the high susceptibility of protein synthesis observed when intact mammalian mitochondria are treated with carbomycin (Kroon and de Vries, 1971; de Vries, 1973; Ibrahim et al., 1974). For example, Ibrahim et al. (1974) found that a 2 μM concentration of carbomycin was sufficient to inhibit protein synthesis by 50%. De Vries et al. (1973) found that intact mitochondria are capable of concentrating carbomycin 30-fold from the medium. This, in part, explains the sensitivity observed when intact mitochondria are used.

The lower sensitivity of mammalian mitochondrial ribosomes to carbomycin and tylosin may reflect an altered peptidyl transferase site in the ribosomes. Lower sensitivities towards other members of the macrolide group are found as well. Erythromycin was a poor inhibitor of CAP binding to rat liver mitochondrial ribosomes (Ibrahim et al., 1974; de Vries et al., 1973). It appears that the binding sites for CAP and the macrolides are not linked in mitochondrial ribosomes as they are in bacterial ribosomes.

Summary of Antibiotic Studies

Mitochondrial ribosomes contain a CAP binding site which is located within the peptidyl transferase locus. They also contain a streptogramin binding site. These sites are known to overlap in E. coli ribosomes. They are thought to overlap in mitochondrial ribosomes as well since their peptidyl transferase activity is inhibited to the same extent as that of bacterial ribosomes when antibiotics from these groups are used.

The lincomycin binding site is distinct from the CAP site. This is supported by the observation that mitochondrial ribosomes respond to CAP in a manner analogous to bacterial ribosomes, but require 100-fold greater concentrations of lincomycin to achieve 50% inhibitory values. Only a 10-fold difference in 50% inhibitory concentration is observed when celesticetin is the drug employed. This indicates that mitochondrial

ribosomes have a binding site for the lincosamines. The reduced sensitivity of the peptidyl transferase activity may be due to one or more of these possibilities: (a) there is a reduced binding affinity for the lincosamines to their site; (b) the binding site is no longer in the correct spatial position for effective inhibition of peptidyl transferase activity; or (c) a conformational change in the ribosome, which may be required for effective inhibition, is not occuring. This study does not discriminate among these possibilities.

The high concentration of the macrolides required to inhibit the mitochondrial ribosomes probably indicates a decrease in the binding affinity of the drugs for their specific sites. This conclusion is supported by the competition studies of de Vries et al. (1973) and Ibrahim et al. (1974), who showed that erythromycin, another macrolide, was not as effective in reversing CAP binding to rat liver mitochondrial ribosomes as it is to yeast mitochondrial ribosomes.

While no bacteria are known to have become CAP resistant through ribosomal mutation, point mutations at this level have been found to induce resistance to the macrolides. These mutants often show cross resistance to lincomycin (Vazquez et al., 1973). These findings may indicate that the macrolide and lincomycin binding sites are not as intimately involved in the

peptidyl transferase locus as is the site of CAP binding. This conclusion is supported by recent studies demonstrating that the CAP binding protein L_{16} is a close neighbor to L_{11}, believed to be crucial to the activity of the peptidyl transferase center (Dietrich et al., 1974). In this connection, it is noteworthy that the mitochondrial ribosomal sites that show the least homology with bacterial ribosomes are the binding sites for the macrolides and lincosamines.

Additional Assays of Mitochondrial Ribosomal Activities

Poly U-dependent phenylalanine polymerizing systems have been used as models for protein synthesis by numerous investigators. These systems mimic the in vivo situation since they require energy, elongation factors, tRNA and a template. However, they differ from the natural system by not requiring initiation factors.

Protein synthesis of mitochondrial ribosomes isolated from fungi has been measured by poly U-dependent systems. Ribosomes isolated from Neurospora mitochondria were reported to be active in this system (Küntzel, 1969a) but at low levels when compared to bacterial systems. High activities, approaching those of E. coli ribosomes, were reported for yeast mitochondrial ribosomes by Grivell et al. (1971a). Functional activities of mammalian mitochondrial ribosomes have been more

difficult to measure with this assay system. Initial observations indicated very low levels (25 to 100 pmoles/mg RNA in 15 min) of phenylalanine incorporation into protein. The difficulty was probably due to nuclease contamination of the mitochondrial ribosomes (de Vries et al., 1971). New methods for the isolation and purification of mammalian mitochondrial ribosomes (O'Brien, unpublished; de Vries et al., 1971) have increased their activity to a maximum of 1100 pmoles/mg in 15 min (Greco et al., 1974), but they still exhibit only a tenth of the activity of E. coli ribosomes in this assay (see Appendix B). The activity measurements obtained for the mitochondrial ribosomes isolated from bovine and rat livers in this study compare well with activities recently obtained by other investigators for rat liver, Xenopus ovaries and Ehrlich ascites cells (Table 10). Rat liver mitochondrial ribosomes are more active in my assays than bovine liver mitochondrial ribosomes not only because they are prepared from younger animals containing more ribosomes per mitochondrion but also because the ribosomes are fresher due to a shorter preparation time. The lack of stability may be due to the combined action of proteases and nucleases on the ribosomes that degrade them during preparation.

Using the poly U-dependent system of Hosokawa et al. (1966) it has been seen that mitochondrial ribosomes are dependent on exogenous elongation factors

TABLE 10

COMPARISON OF THE ACTIVITIES OF 55S MITOCHONDRIAL RIBOSOMES ISOLATED FROM VARIOUS ORGANISMS IN POLY U-DEPENDENT REACTIONS

Investigator	Organism	Incorporation of [^{3}H]Phe pmoles/mg RNA in 15 min
Denslow	cow	561
	rat	850
Leister	Xenopus	503
Greco	rat	1090
Ibrahim	rat	980
de Vries	rat	25
Avadhani	mouse (Ehrlich ascites)	368

References:
Leister and Dawid, 1974
Greco et al., 1973
Greco et al., 1974
Ibrahim et al., 1974
Avadhani and Rutman, 1974

and that they are able to use either bacterial or mitochondrial elongation factors. It has been determined from the GTP binding study that near stoichiometric quantities of an EF-G type factor are bound to the isolated mitochondrial ribosomes. This factor can be removed only when the ribosomes are washed under stringent conditions (Table 9). The crude factors which are added to support the poly U-dependent reaction therefore are probably bringing in EF-T factors rather than EF-G factors.

The requirements by E. coli ribosomes for elongation factors is met by a protein concentration of 0.1 mg/ml, which is adequate to saturate these ribosomes (Appendix A). On the other hand, 8-fold higher levels of bacterial factors were needed to obtain activity with mitochondrial ribosomes. The relatively high amounts of heterologous factors that are needed to support the activity of the mitochondrial ribosomes probably reflects significant differences in the ability of each ribosome to bind the bacterial elongation factor T. It is remarkable that mitochondrial ribosomes are able to use bacterial factors at all, even at these high levels, since they exhibit pronounced physical-chemical differences when compared to E. coli ribosomes. The routine use of mitochondrial elongation factors is complicated by their high lability (Grandi and Küntzel, 1970) and the presence of inhibitors of protein synthesis (probably nucleases) in the crude preparations (Appendix B). In yeast mitochondrial factor

preparations, Richter and Lipmann (1970) observed that EF-T was more labile than EF-G. The mammalian mitochondrial EF-T factor may be labile as well.

By analogy to E. coli, GTP binding by mitochondrial ribosomes is probably mediated by an elongation factor having properties similar to EF-G (Nishizuka and Lipmann, 1966; Bodley et al., 1970). This factor appears to be bound to mitochondrial ribosomes in near stoichiometric amounts. The mitochondrial EF-G appears to be more tightly bound to mitochondrial ribosomes than the bacterial factor is to E. coli ribosomes. EF-G is easily removed from bacterial ribosomes by washing them with 1 M NH_4Cl. Quantitative removal can be accomplished after two to three washing steps. Quantitative removal of the factor from mitochondrial ribosomes can be accomplished only with 2 M NH_4Cl. The tighter binding of EF-G by mitochondrial ribosomes may explain the stability of the mitochondrial ribosome-factor-[^{3}H]GTP complex in the absence of fusidic acid. The bacterial complex, on the other hand, is very unstable unless fusidic acid is present in the reaction mixture (Richter et al., 1971).

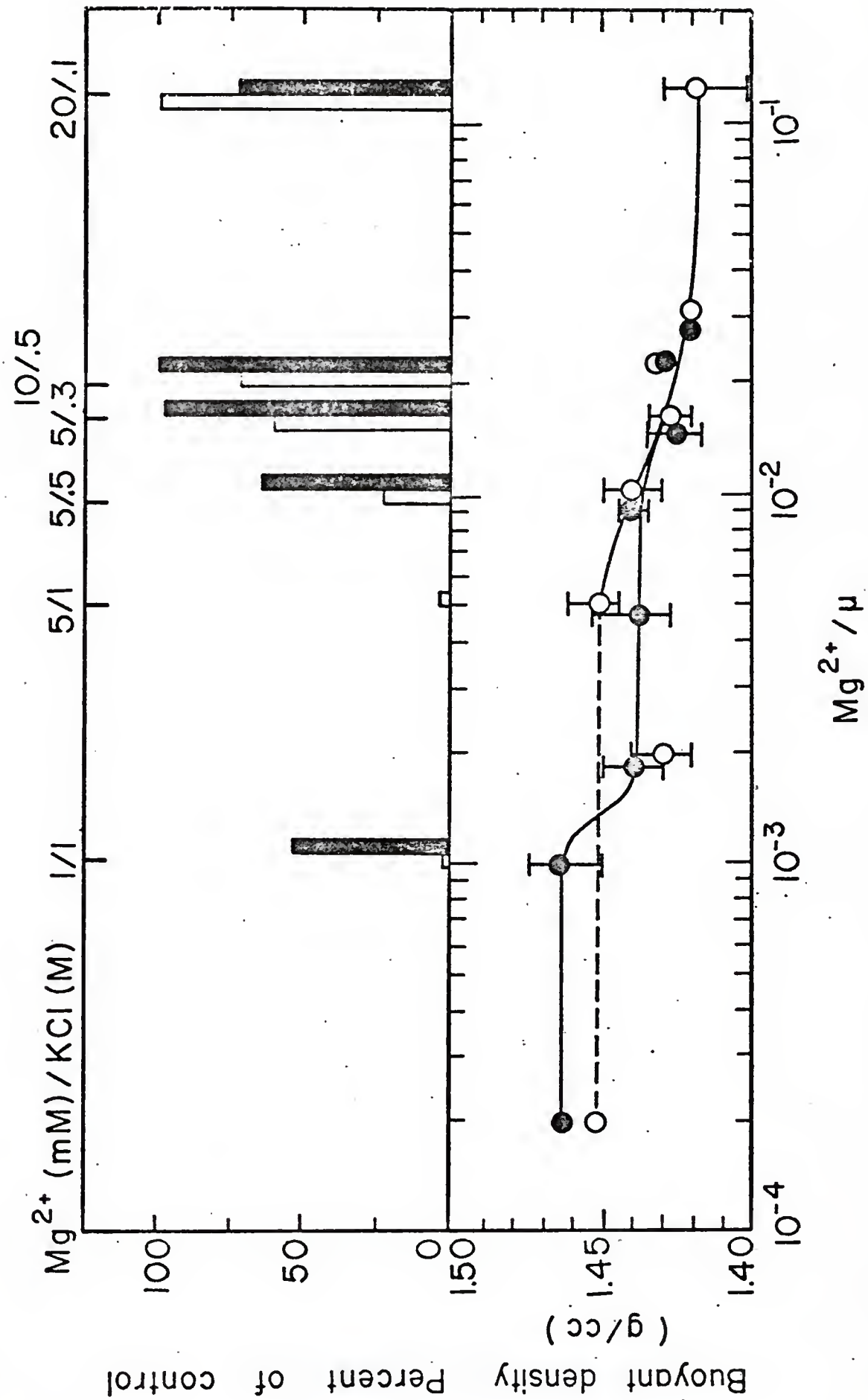
Stability of the Peptidyl Transferase Locus and [^{3}H]GTP Binding Ability

Mitochondrial ribosomes were washed with buffers of compositions known to remove mitochondrial ribosomal proteins. In one series of experiments, the object was to remove a group of proteins intimately involved in

the peptidyl transferase activity of the ribosomes. Conditions, adequate to remove a small group of ribosomal proteins, were found to abolish peptidyl transferase activity. It was not determined whether the ribosomal proteins removed are in the peptidyl transferase locus or whether they are merely required indirectly to stabilize this site. In another series of experiments, the object was to examine mitochondrial ribosomes for a bound factor analogous to elongation factor G found in other ribosomal systems which is responsible for GTP binding. It was established that mitochondrial ribosomes do contain a GTP binding factor. Conditions which are adequate to remove this factor from the ribosomes were found.

In other systems, it has been demonstrated that ribosomes are generally stable to washing in buffers that contain moderate amounts of salt provided the Mg^{2+} level does not drop below certain critical values (Staehelin et al., 1969). Optimum Mg^{2+} levels are specific for each kind of ribosome. The stability of mitochondrial ribosomes under various Mg^{2+} and salt conditions has been studied by O'Brien (personal communication). After washing with each buffer, the net protein content of the resulting ribosomal core particles was measured by their buoyant density in CsCl gradients. It was observed that the buoyant density increases with increasing Mg^{2+}/μ ratios (Figure 12), indicating a loss of protein from the subribosomal particles.

Figure 12. Comparison of functional activities and buoyant densities of mitochondrial ribosomes prepared under buffer conditions of decreasing Mg^{2+}/μ ratios. Buoyant densities of mitochondrial particles were obtained by O'Brien (unpublished data) by fixing subribosomal particles (o——o 39S, and ●——● 28S) with 5% formaldehyde and centrifuging to equilibrium in CsCl gradients containing 0.3% formaldehyde. Peptidyl transferase ▭ and [^{3}H]GTP binding ▬ activities were measured as described in "methods."


Buoyant density Percent of control
(g/cc)
1.40
1.45
1.50
0
50
100
Mg2+ (mM) / KCl (M)
1/1
5/1
5/5
5/3
10/5
20/1
Mg2+ / μ
10-4
10-3
10-2
10-1

Conditions that do not disrupt functional activities but are adequate to remove adsorbed non-ribosomal proteins from other kinds of ribosomes (Nishizuka and Lipmann, 1966) do disrupt the peptidyl transferase activity of mitochondrial ribosomes. When we use buffer* Z (5 mM $MgCl_2$ and 0.5 M KCl) the peptidyl transferase activity of mitochondrial ribosomes is reduced by 75% whereas that of E. coli ribosomes remains stable (Table 2). These results indicate that mitochondrial ribosomes require a higher Mg^{2+} concentration than bacterial ribosomes to maintain functional activity. This is supported by the additional observation that mitochondrial ribosomes are relatively stable when they are washed in buffer BTR (10 mM $MgCl_2$ and 0.5 M KCl). When the $MgCl_2$ concentration is kept at 5 mM and the KCl concentration is raised to 1M, as in buffer Y, the peptidyl transferase activity is abolished. This occurs at the same time that a group of 9 proteins are washed from the large ribosomal subunit. It is not clear whether the peptidyl tranferase diminution is due primarily to the stripping of these proteins; that is, whether these proteins contribute directly to the structure of the peptidyl transferase locus, or whether the ribosome structure itself has become so destabilized by the low Mg^{2+} and high salt concentration so as to distort the important steriochemistry of the center.

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* For complete buffer compositions see "methods."

Buffer NCB (10 mM $MgCl_2$ and 1 M NH_4Cl) which effectively removes EF-G from E. coli ribosomes (Table 9 and Nishizuka and Lipmann, 1966; Bodley et al., 1970) begins to remove an analogous factor from mitochondrial ribosomes. The mitochondrial factor, therefore, appears to bind more tightly to mitochondrial ribosomes than does the corresponding bacterial EF-G to E. coli ribosomes. This is supported by the additional observation that fusidic acid, which is essential to stabilize the bacterial ribosome-G factor-GTP complex does not seem to be required by the mitochondrial system.

The GTP binding ability of mitochondrial ribosomes is harder to disrupt when buffers containing KCl are used instead of those containing NH_4Cl (Figure 12). GTP binding activity is diminished by 36% in buffer Z (5 mM $MgCl_2$, 0.5 M KCl) and by 47% in buffer A (1 mM $MgCl_2$, 1 M KCl). That NH_4Cl is more effective than KCl in dissociating ribosomal proteins from mitochondrial ribosomes also has been observed for bacterial ribosomes (Spitnik-Elson and Atsmon, 1969). It is surprising that it is easier to disrupt the peptidyl transferase activity of mitochondrial ribosomes than it is to remove bound elongation factor G. Thus it appears that the peptidyl transferase activity of mitochondrial ribosomes is rather labile.

Conclusions

1. 55S mitochondrial ribosomes isolated from rat and bovine liver are active in protein synthesis as

tested by the model poly U dependent reaction and two partial reactions of protein synthesis: peptidyl transferase and [^{3}H]GTP binding. Therefore, these particles are the functional ribosomes rather than artifacts produced during the isolation procedure.

2. Overall mitochondrial ribosomal function is similar to that of other ribosomes. The peptidyl transferase activity is localized on the large subunit. Like other ribosomes, they require, in addition to energy and amino acids, a template, tRNA and soluble factors for protein synthesis.

3. On the basis of near stoichiometric binding of [^{3}H]GTP by isolated mitochondrial ribosomes, it seems likely that they contain a bound factor having properties similar to those of bacterial EF-G.

4. The peptidyl transferase locus of mitochondrial ribosomes is similar to the bacterial locus when probed with chloramphenicol and the streptogramins. Therefore, mitochondrial ribosomes, like bacterial ribosomes, contain binding sites for chloramphenicol and the streptogramins within the peptidyl transferase locus.

5. When the mitochondrial ribosomes are probed with the lincosamines or the macrolides, diminished susceptibilities of peptidyl transferase activity relative to E. coli ribosomes are observed. A less efficient binding of the macrolides to the mitochondrial ribosome is supported by the 300- to 700-fold greater concentrations required to inhibit the reaction by 50% when compared to E. coli ribosomes. Additional support comes from experiments indicating a diminished ability of the macrolides to compete for CAP binding to rat liver mitochondrial ribosomes (Ibrahim et al., 1974; de Vries et al., 1973). The diminished susceptibility of mammalian mitochondrial ribosomes to the lincosamines may reflect either a diminished binding affinity for these drugs or an altered spatial configuration at the peptidyl transferase locus so that these drugs can no longer interfere effectively with the peptidyl transferase activity.

6. Pronounced differences between mammalian mitochondrial ribosomes and those from the ascomycete yeast do exist with respect to their susceptibility to inhibitors specific for the peptidyl transferase locus. The mitochondrial ribosomes of Neurospora, another ascomycete, resemble the mammalian mitochondrial ribosomes in their diminished sensitivity to the macrolides (de Vries et al., 1973). There-

fore, it is still not clear if phylogenetic differences occur systematically.

New Directions in Research

Partial reconstitution experiments in combination with affinity labeling experiments may resolve the fine structure of the peptidyl transferase locus of mitochondrial ribosomes. Several substrate analogues, as well as antibiotic analogues, are now available for affinity labeling of ribosomal proteins within this locus on *E. coli* ribosomes. These probes may also covalently bind to the ribosomal proteins of mitochondrial ribosomes. Two-dimensional polyacrylamide gel electrophoresis analysis of the labeled proteins may be useful in identifying those proteins that belong to the peptidyl transferase site.

The fine structure and function of mitochondrial ribosomes can be explored further by examining other activity centers for homologies with bacterial ribosomes. In particular, a detailed study of the translocation center may prove of value. The interchangeability of the bacterial elongation factor EF-G for the mitochondrial [^{3}H]GTP binding factor should be investigated. This could be accomplished by observing whether the bacterial EF-G can mediate the binding to [^{3}H]GTP to mitochondrial ribosomes washed free of the GTP-binding factor. Since bacterial factors can support poly U directed protein

synthesis, it seems likely that these factors should be exchangeable. However, as our studies of the [^{3}H]GTP binding ability of mitochondrial ribosomes indicate, near stoichiometric amounts of an EF-G like factor are found bound to the ribosomes even when these are washed with high salt concentrations. Therefore, it is not absolutely clear that mitochondrial ribosomes can use bacterial EF-G.

The bacterial EF-G binding site consists mainly of the L_7 and L_{12} ribosomal proteins which can be removed from the ribosomes by treatment with 1 M NH_4Cl and 50% ethanol. Mitochondrial ribosomes should be examined to see whether proteins of similar properties and function can be obtained. Reconstitution experiments with homologous and heterologous ribosomal proteins belonging to this site could then be investigated. Antibiotic probes of translocase activity are also available; the more interesting ones being fusidic acid and thiostrepton, since these may interact with mitochondrial ribosomes.

Antibodies to individual E. coli ribosomal proteins have been prepared by Witmann's group (Stöffler and Wittmann, 1971). These have been used to determine the functional involvement of several ribosomal proteins in protein synthesis. Homologies with the mitochondrial system could be investigated by using E. coli specific antibodies to block functional steps in the mitochondrial system. In particular these probes would be useful in the assays of peptidyl transferase and [^{3}H]GTP binding.

APPENDIX A
CALCULATIONS

The Quantitative Effect of Cytoplasmic 60S Subribosomal Particles on the 55S Peptidyl Transferase Activity

Mitochondrial ribosomes, prepared by procedure 1, were purified by sucrose density gradient centrifugation in buffer T as described. The fraction containing the 55S ribosomes also contained a small but variable (5 to 15%) amount of cytoplasmic 60S particles (Figure 2). The specific activity of the 60S particles in the peptidyl transferase assay was only 10 to 15% that of mitochondrial ribosomes. Therefore, the 60S particles contributed only 2 to 3% of the measured 55S ribosomal activity.

Quantitation of Ribosomes

E. coli, bovine liver mitochondrial and cytoplasmic ribosomes were quantitated as follows:

1 A_{260} *E. coli* 70S = 23 pmoles

1 A_{260} *E. coli* 50S = 33 pmoles

1 A_{260} mitochondrial 55S = 32 pmoles

1 A_{260} mitochondrial 39S = 55 pmoles

1 A_{260} mitochondrial 28S = 84 pmoles

1 A_{260} cytoplasmic 80S = 17 pmoles

1 A_{260} cytoplasmic 60S = 26 pmoles

Amounts of ribosomes and subunits were estimated spectrophotometrically at 260 nm assuming that $E^{1\%}_{1cm}$ = 160 for E. coli 70S, $E^{1\%}_{1cm}$ = 135 for bovine liver cytoplasmic ribosomes, and $E^{1\%}_{1cm}$ = 110 for bovine liver mitochondrial ribosomes. The molecular weights for the monosome and subunits of each type of ribosome are as follows:

E. coli	70S = 2.7×10^6 daltons
	50S = 1.9×10^6 daltons
	30S = 0.8×10^6 daltons
Bovine liver cytoplasmic	80S = 4.5×10^6 daltons
	60S = 3×10^6 daltons
	40S = 1.5×10^6 daltons
Bovine liver mitochondrial	55S = 2.83×10^6 daltons
	39S = 1.65×10^6 daltons
	28S = 1.18×10^6 daltons

Quantitation of [^{3}H]GTP Used in the GTP-Binding Assay

[8-^{3}H]GTP, specific activity 12 Ci/mmole was obtained from Schwartz-Mann. The purity of the radioactive label was determined in order to quantitate the binding of [^{3}H]GTP to ribosomes. A 5 µl sample was applied to a PEI-cellulose thin layer plate. The technique of Randerath and Randerath (1967) which involves a stepwise elution with 0.2 M LiCl, 1.0 M LiCl, and 1.6 M LiCl was used. This method detects 10 to 20 nmoles of material. The plate was calibrated with GMP and GTP standards containing 10 to 20 nmoles. To determine the composition

of the labeled material, the PEI-cellulose was scraped in 0.5 cm sections from the plate and counted in a liquid scintillation counter. 41% of the material migrated as GTP and 10% as GDP. Both GTP and GDP bind to the ribosome to give the complex described in "results." These factors were included in calculations to determine the binding of GTP to ribosomes (Table 9).

APPENDIX B
OPTIMIZATION OF THE POLY-U-DEPENDENT POLYMERIZATION OF [^{3}H]-PHENYLALANINE

Starting with the basic reaction mixture of Hosokawa (1966), the poly U dependent system was optimized for tRNA and poly U levels using E. coli ribosomes. Over the range from 100 µg/ml to 1 mg/ml, the optimum level of tRNA was found to be 0.5 mg/ml. In the same manner, poly U was varied from 160 µg/ml to 800 µg/ml and the optimum quantity was 640 µg/ml.

The E. coli reaction was stimulated 3-fold by the addition of 33 mM NH_4^+ when an ammonium sulfate suspension of pyruvate kinase was used. This type of stimulation by NH_4^+ ions is well known (Miskin et al., 1970). However, in the case of mitochondrial ribosomes, greater activity was observed when no NH_4^+ ions were introduced into the reaction mixture (Table 11). Grivell has noted that 33 mM NH_4^+ inhibited yeast mitochondrial ribosomes by 75% and NH_4^+ levels in excess of 125 mM by 90% (Grivell et al., 1971a). It is possible that mammalian mitochondrial ribosomes are sensitive to NH_4^+ ions as well.

E. coli factors prepared by the method of Nirenberg (1963) were very active in the E. coli system. Figure 13 illustrates a saturation curve obtained with a poly U dependent reaction for a typical factor preparation.

TABLE 11

POLY PHENYLALANINE POLYMERIZING ACTIVITY OF RIBOSOMES IN THE PRESENCE OF NH_4^+ IONS

Ribosomes	$[NH_4^+]$	Activity pmoles/mgRNA in 15 min*
70S	-	3,965
70S	33 mM	8,085
55S	-	850
55S	33 mM	375
28S+39S	-	1,624
28S+39S	33 mM	1,088

* The results are corrected for the minus ribosome controls for each system: 120 pmoles/mgRNA in 15 min for the experiment using 70S ribosomes; 150 pmoles/mgRNA in 15 min for the experiments using 55S and 28S+39S mitochondrial ribosomes. E. coli ribosomes were washed with a buffer containing 1 M NH_4Cl. Mitochondrial ribosomes were prepared through sucrose density gradients in T buffer. 1.2 A_{260} units of 70S ribosomes, 0.5 A_{260} units of 55S ribosomes, 0.3 A_{260} units 39S, and 0.3 A_{260} units of 28S ribosomes were used. The experimental conditions are described in "methods."

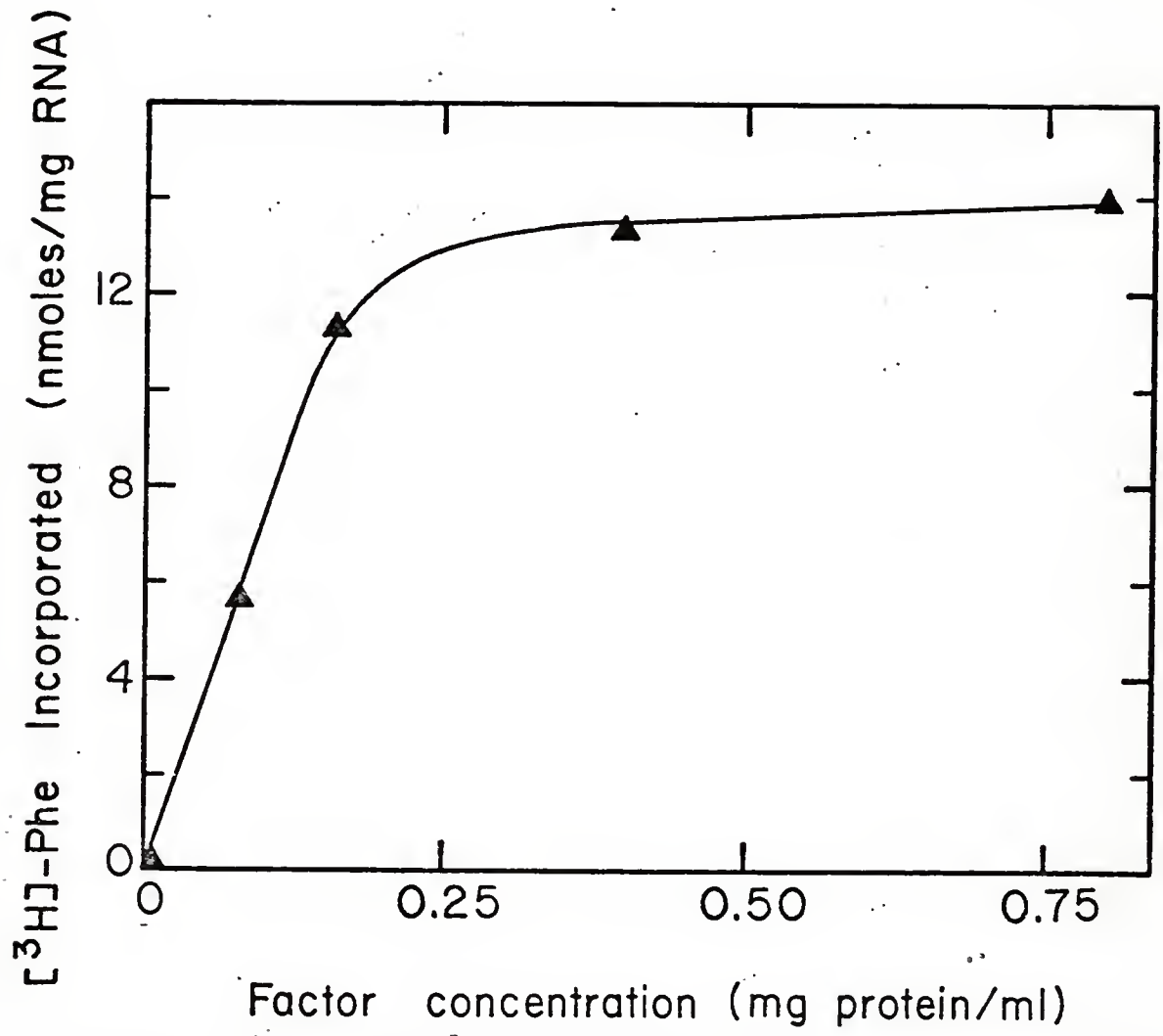
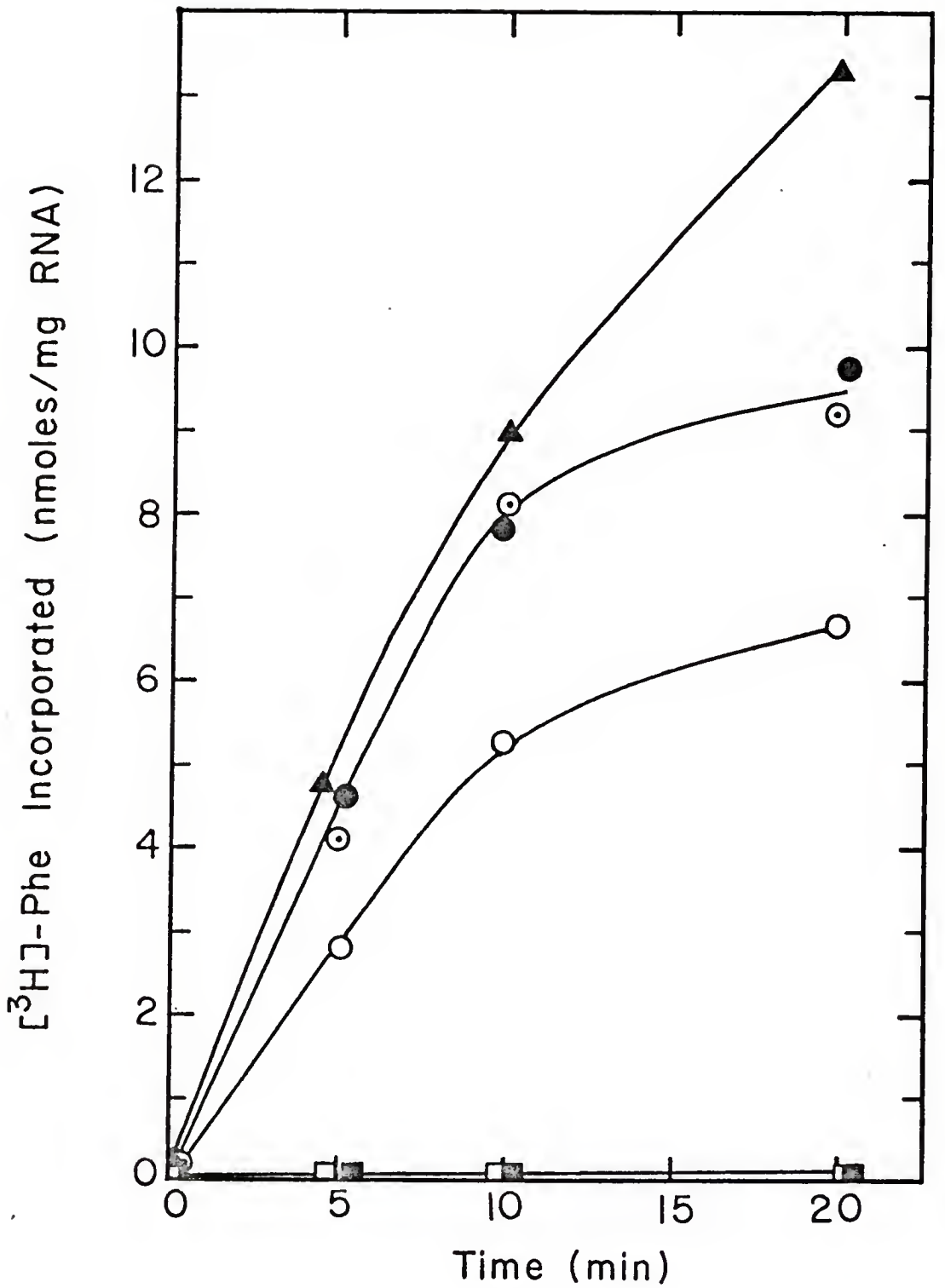


Figure 13. Synthesis of polyphenylalanine by E. coli ribosomes in the presence of increasing concentrations of E. coli factors. Assay conditions are found under "methods." The concentration of E. coli factors was varied as indicated. Each reaction vessel contained 1.17 A_{260} units of E. coli ribosomes.

Although 0.43 mg/ml E. coli factors were sufficient for maximum activity with E. coli ribosomes, at least 1 mg/ml factors was necessary to stimulate the mitochondrial system. This requirement was also observed by Ibrahim et al. (1974). Inhibitors to the poly U dependent system, which may be nucleases, were routinely found in the preparation of mitochondrial factors. The effect of these inhibitors on an optimal E. coli system are depicted in Figure 14. As can be seen, the addition of mitochondrial factors to a system containing saturation levels of E. coli factors suppresses the incorporation of [^{3}H]Phe by E. coli ribosomes.

Figure 14. Determination of an inhibitor of protein synthesis in preparations of mitochondrial elongation factors. The reaction conditions for the poly-U-dependent polyphenylalanine synthesizing system are found in "methods." Each reaction vessel contained 1.17 A_{260} units of E. coli 70S ribosomes and 0.43 mg protein/ml E. coli elongation factors. ▲——▲, control reaction containing only E. coli elongation factors; ●——●, addition of 0.2 mg/ml mitochondrial factors A prepared as described in "methods"; ⊙——⊙, addition of 0.2 mg/ml mitochondrial factors B prepared as described except that the 14,000 x g supernatant was not adjusted to 0.5 M KCl; o——o, addition of 0.2 mg/ml mitochondrial factors C which was the combined lower 1/4 fraction from the 230,000 x g supernatant of mitochondrial factors A and B.


[3H]-Phe Incorporated (nmoles/mg RNA)
0
2
4
6
8
10
12
0
5
10
15
20
Time (min)

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I certify that I have read this study and that, in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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